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ADVANCES IN INSECT PHYSIOLOGY

Volume 1

Waldo E. Cohn

Advances in

INSECT PHYSIOLOGY

VOLUME 1

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Advances in
Insect Physiology

Edited by

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VOLUME 1

1963

ACADEMIC PRESS
London and New York

ACADEMIC PRESS INC. (LONDON) LTD.
BERKELEY SQUARE HOUSE
LONDON, W.1

U.S. Edition published by

ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK 3, NEW YORK

Library of Congress Catalog Card Number: 63-14039

Printed in Great Britain by Spottiswoode, Ballantyne & Co. Ltd.,
London and Colchester

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Preface

Explosive periods of evolution are well known to paleontologists who study the fossil record. The biological sciences are in the midst of such an evolutionary phase at the present time; and the physiology of insects is enjoying a full share of this expansion.

During the past twenty years the insects have become increasingly recognized as an ideal medium for the study of all aspects of physiology. The chemistry of natural products, the dynamic biochemistry of intermediary metabolism, the fine structure and biophysics of cells and their parts, electrophysiology of nerve and muscle, ionic and osmotic regulation, physiological genetics and "Entwicklungsmechanik", the rôle of hormones and neurohumours, the mechanism of sensory perception, the physiological basis of behaviour, and the contribution of all this physiological knowledge to the understanding of ecology—these are some of the fields of enquiry in which the insects are being used to an ever-increasing extent.

By and large, the subject of insect physiology has been well served by reviews. But there is still room for a reviewing journal in which an opportunity will be given for the comprehensive survey of selected topics which happen to be the most active growing points of the subject. It will be the policy of this series to interpret insect physiology in the broad terms set out above. It is to be hoped that reviews of the kind we aim to publish will serve not only as a guide to recently acquired knowledge, but as a stimulus and catalyst for further advances.

J. W. L. B.

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March, 1963 V. B. W.

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I. INTRODUCTION

In insects evidence concerning the regulation of physiological function by hormones is well established. Indeed, the studies upon neurosecretion and metamorphosis are unequalled in the field of comparative endocrinology. However, the term neurosecretion probably encompasses other physiological phenomena in insects, for, as aptly described by Burgen (1959), it can be defined as the production of physiologically active substances within neurones which may act either locally on neighbouring neurones or effector cells or be carried in the blood to act on more distant sites. Over the past decade evidence has been accumulating that insects contain a number of neurosecretory substances distinct

from those involved in growth and metamorphosis. The best known of these is acetylcholine, which in mammals has been linked with physiological mechanisms of nerve transmission. It is the purpose of this chapter to describe in detail what is known about the acetylcholine system in insects and, more briefly, to describe the possible occurrence of other pharmacologically active substances, the identity and physiological function of which are even less certain. First let us deal with the history of acetylcholine and the events that led to its ascribed function in mammals.

The speculations of Claude Bernard and the experiments of DuBois-Reymond in the late nineteenth century about junctions between nerve cells and other excitable tissues, together with the brilliant hypothesis of Elliott (1905) and the pharmacological observations of Sir Henry Dale and co-workers in the early decades of the present century set the stage for the classical experiments of Loewi (1921). Unequivocal evidence was obtained that a chemical substance which controlled organ function was released from a nerve ending. Thus was born the neurohumoral or neurotransmitter properties of acetylcholine, (β-acetoxyethyltrimethylammonium hydroxide), first synthesized by Baeyer (1867). Following the experiments of Loewi an overwhelming number of physiological, biochemical and histochemical investigations have been carried out to establish the significance of acetylcholine in nervous and neuromuscular activity. At present there is strong evidence that acetylcholine acts as a spatial, temporal link at vertebrate skeletal neuromuscular junctions, between nerve cells of autonomic ganglia and at their post-ganglionic nerve endings (parasympathetic and some sympathetic). The evidence that acetylcholine plays a similar role in central nervous tissue is not as conclusively proven and controversies have arisen concerning the purpose of the ester in the passage of excitation along membranes of axons and muscle fibres. In summary it can be said that in certain parts of the vertebrate nervous system acetylcholine acts as a chemical mediator of nervous activity. In essence the substance is thought of as a neurotransmitter agent.

In nature, acetylcholine or associated enzymes are not the exclusive property of nervous tissue. For example, a number of plants contain significant amounts of acetylcholine (Lin, 1957), while in the vertebrates the intestinal mucosa (Feldberg and Lin, 1960) and placenta (Comline, 1946) are capable of synthesizing high amounts of the substance. Synthesis of acetylcholine can be obtained with a micro-organism *Lactobacillus plantarum* (Keil and Kritter, 1935) and a recent report shows that an alga, *Nitella flexilis*, contains acetylcholinesterase

(Dettbarn, 1962). Because of its wide distribution there should be no reason to doubt that the acetylcholine system participates in neural activity. In fact, the substance may influence cell function in a number of ways. These observations apply to the occurrence of acetylcholine and other pharmacologically active substances in insects for they are found in abundant quantities in tissues of neural and non-neural origin.

II. Characteristics of the Acetylcholine or Cholinergic System in Insects

Although the purpose of this chapter is to discuss the physiological significance of acetylcholine (ACh) in insects, two enzymes, acetylcholinesterase (AChE) and choline acetylase (ChA), are known to play a part in physiological activity. A hypothetical picture of the role of these three elements, together defined as the acetylcholine or cholinergic system, would be the occurrence of ACh and ChA in a preganglionic neuron and the presence of AChE in or near the membrane of an adjacent postganglionic neurone. ACh is released at the preganglionic nerve ending to momentarily depolarize the membrane of the postganglionic nerve cell and then it is hydrolysed by AChE. The products of hydrolysis, acetate and choline would be used for resynthesis of ACh in the preganglionic nerve ending. Physical events of nerve conduction (resting and action potential) would comprise a fourth component of the system. Thus detection of the cholinergic system in a ganglion or neuromuscular apparatus of an insect would suggest transmission of nervous activity, while the occurrence of the ester alone in non-nervous tissue or secretion of a gland would indicate an entirely different type of function. To obtain a picture of where ACh might have physiological effects in insects the distribution of the ester has been listed in Table I. Where possible the other elements of the cholinergic system have been included. The data for ChA are reasonably complete but those for AChE are mainly subjective to the presence of ACh. The evidence for ACh in Table I is not unequivocal identification. This aspect is fully discussed in the text.

A. Identification of Acetylcholine

The first finding of ACh in insects can be attributed to Gautrelet (1938) for bees and to Corteggiani and Serfaty (1939) for eight different species of insects. The information, including evidence for the presence of AChE, showed that nervous tissue contained ACh. In an attempt to demonstrate the physiological function of ACh in insects a remarkable

TABLE I

Occurrence of elements of the cholinergic system in insects

Class and Species	Tissue	Amounts			Authors
		ACh*	AChE†	ChA‡	
ORTHOPTERA					
Periplaneta americana	Head	8	+		Corteggiani and Serfaty (1939)
	Nerve cord	70	+		Mikalonis and Brown (1941)
	Nerve cord	46		+	Tobias *et al.* (1946)
	Thoracic ganglia	58			
	Connectives	33			
	Nerve cord	32			Roeder (1948)
	Nerve cord	+			Chang and Kearns (1955)
	Head	9			Lewis and Smallman (1956)
	Thoracic nerve cord	50			
	Nerve cord	66—75			Smallman and Fisher (1958)
	Head	15			Colhoun (1958a, c; 1959a)
	Brain	143	137	50	
	Brain + suboesophageal ganglion	135	153	53	
	Ventral nerve cord	63	270	10	
	Thoracic nerve cord	79	221	11	
	Thoracic ganglia	95	331	20	
	Thoracic connective	31	238	2	
	Abdominal nerve cord	65	187	6	
	6th abdominal ganglia	63	314	18	
	5th leg nerve	1·2 per 60	176	2	
	Cercal nerves	1·4 per 60	150	3	
	Coxal muscle	0	0	0	
	Flight muscle	0	?	0·08	
	Heart	0	0	0	
	Blood serum	0	?	0	
	Blood cells	0	?	0	

TABLE I—*continued*

Class and Species	Tissue	Amounts			Authors
		ACh*	AChE†	ChA‡	
Gryllus domesticus	Head	7—10	+		Corteggiani and Serfaty (1939)
	Brain	65	+		
Schistocerca gregaria	Head	60		+	Bellamy (1958)
HEMIPTERA					
Oncopeltus fasciatus	Eggs	4—201	+[a]	+	Mehrotra (1960a)
					[a]Smith and Wagenknecht (1956)
LEPIDOPTERA					
Chilo simplex	Eggs	1—3mg	+[b]	600[c]	Yushima and Chino (1953)
					Chino and Yushima (1953)
					[b]Chino and Yushima (1954)
Barathra brassicae	Eggs	1—8 mg	+	500[c]	[c]Yushima (1957)
Schoenbius incertulas	Eggs	+			Yushima (1956a)
Sidemia depravata	Eggs	1 mg			Yushima (1956b)
Polia illoba	Eggs	0·5 mg			
Euxoa segetis	Eggs	1 mg			
Bombyx mori	Eggs	140	+	+	Chino (1957)
Sanninoidea exitiosa	Eggs	20[d]	+		Smith and Wagenknecht (1956)
					[d]Colhoun (unpublished data)
Platysamia cecropia	Diapausing brains	+	None	+	Van Der Kloot (1955)
	Developing brain	+	+	+	
	Ganglia	+			
	Larvae		+		
	Adult brain	+	+	+	
Telia polyphemus	Diapausing brain		None		Van Der Kloot (1955)
Samia walkerii	Diapausing brain		None		
Galleria sp.	Pupal brains		+		
Arctia caja	Hypertrophied				
	Silk gland	4 mg			Morley and Schachter (1961)
	Cocoon	300			

TABLE I—*continued*

Class and Species	Tissue	Amounts ACh*	AChE†	ChA‡	Authors
Arctia caja	Larval blood	4—9			Morley and Schachter (1962)
	Male accessory sex gland	+			
	Male ejaculatory duct	2—5 mg			
	Eggs	300			
	Female bursa copulatrix	8 mg			
Arctia villica	Male reproductive system	400			
Spilosoma lubnicipeda	Abdomen	80—100			
Salutea sp.	Abdomen	10—15			
Panaxia dominula	Abdomen	+			
Lygaena lonicerae	Abdomen	300—500			
Laothoe populi	Body	+			
Smerthinus ocellatus	Body	+			
Meristro trigrammica	Body	+			
Agrotis exclamationis	Body	+			
Plutella maculipennis	Body	+			
Pieris brassicae	Eggs	0·9—13	+		David (1959)
COLEOPTERA					
Leptinotarsa decemlineata	Head	3—10	+		Corteggiani and Serfaty (1939)
Carabus auratus	Head	45—55	+		
Carausius morosus	Head	8—9	+		
	Nerve cord	100—200	+		
Tenebrio moliter	Head	20—35	+[e]	+	Lewis and Smallman (1956) [e]O'Brien (1953)
HYMENOPTERA					
Apis mellifera	Head	+	+		Gautrelet (1938)
	Brain		+		Metcalf and Marsh (1949)
Xylocopa violacea	Brain	200			Corteggiani and Serfaty (1939)
Apis mellifera	Head	9			Augustinsson and Grahn (1954
	Honey	+			Marguardt and Vogg (1952)

TABLE I—*continued*

Class and Species	Tissue	Amounts			Authors
		ACh*	AChE†	ChA‡	
Apis mellifera	Honey	2			Goldschmidt and Burkert (1955)
	Royal jelly	800			Henschler (1956)
	Food for types of bee larvae	+			Henschler and Rhein (1960)
	Royal jelly	683			Colhoun and Smith (1960)
Vespa crabro	Venom	50 mg			Bhoola *et al.* (1961)
DIPTERA					
Musca domestica	Whole fly	47			Tobias *et al.* (1946)
	Head	1·5			Chefurka and Smallman (1956)
	Head	26	2700[f]	6[g, h]	Lewis and Smallman (1956) [g]Frontali (1958) [h]Colhoun (1958c) [h]Mehrotra (1961) [f]Smallman (1958)
	Brain	80			Colhoun (1958a)
	Head	44			
	Eggs	4—488	+	+	Mehrotra (1960)
Lucilia sericata	Whole insect	1		+	Lewis (1953)
	Head	6·3			
	Thorax	1			
	Abdomen	< 0·25			
Caliphora erythrocephata	Whole insect	1		+	Lewis (1953)
	Head	6·4			
	Head	32·7	2500[i]	900[k]	Lewis and Smallman (1956) [i]Smallman (1958) [k]Smallman (1956)
	Brain estimated	500			Lewis and Smallman (1956)

* ACh = acetylcholine in μg per gram tissue, wet or dry weight.
† AChE = Acetylcholinesterase activity as mg ACh hydrolysed/g tissue/hr.
‡ ChA = Choline acetylase activity as mg ACh synthesized/g tissue/hr.

series of experiments were performed by Mikalonis and Brown (1941). Nerve cords were isolated from *Periplaneta* and treated with eserine. Measurements of the ACh content of the cords were made and the results showed an increase in ACh which seemed to be correlated with electrical nervous activity of the isolated nerve cords. These experiments were rather similar to those demonstrating function of ACh in cervical ganglion of cat (Feldberg and Vartianen, 1935). With another species of Orthoptera, Means (1942) obtained evidence of the occurrence of AChE in various parts of the nervous system. The highest enzyme activity was found in the brain, ganglia and ventral nerve cord. The experiments of Tobias *et al.* (1946) indicated that synthesis of ACh occurred in extracts of thoracic nerve cord of *Periplaneta*. Thus, within a period of 8 years, evidence had accumulated that the nervous tissues of insects contained the essential components of the cholinergic system and possibly participated in nervous conduction.

However, the results of Tobias *et al.* (*loc. cit.*) showed that ACh increased in nervous tissue of *Periplaneta* poisoned by DDT. DDT did not inhibit AChE so an anomaly was evident in a correlation between inhibition of AChE and ACh. Roeder (1948), investigating synaptic and axonic transmission in nerve cords of *Periplaneta*, concluded that there was no evidence showing that ACh was a mediator of nervous activity, although anticholinesterases (a number of organophorus esters) disrupted synaptic transmission in the sixth abdominal ganglion. The conclusion was also based upon the failure of high concentrations of ACh and other choline esters to interfere with transynaptic conduction when applied to the ganglion. Pretreatment with DFP or HETP (impure TEPP) did not potentiate an effect of ACh.

Hopf (1952), in seeking an explanation for the mode of action of organophosphorus compounds, stated that the evidence of Tobias and his co-workers only indicated the presence of a substance which acted like ACh when tested upon the rectus abdominis muscle of the frog. Like Roeder (1948) Hopf was unable to obtain effects with externally applied ACh. The occurrence of ACh in insects was also doubted by Prosser (1952) who pointed out that the nervous tissue of cockroaches contained an analogue of ACh which was active upon Arthropod preparations which were exceedingly insensitive to ACh. Thus uneasiness was felt about the identity of ACh in insects and it would seem that these doubts had their effect upon subsequent workers, for a series of papers appeared which culminated in reasonable and unequivocal identification of ACh.

Lewis (1953) obtained evidence by use of partition chromatography

that two species of blowflies contained ACh. Within a year Augustinsson and Grahn (1954) showed that heads of bees contained an ester with chemical and pharmacological properties of ACh. Two unknown choline esters were also detected. Chefurka and Smallman (1955 and 1956) subjected extracts of heads of houseflies to electrophoresis, chromatography, infra-red spectroscopy and chemical and pharmacological assay. The pharmacologically active substance was identified as ACh. For nervous tissue of *Periplaneta* Chang and Kearns (1955) showed that ACh was the only choline ester present. These results were

TABLE II

Reports of other choline esters in insects

Class and Species	Tissue	Choline Ester	Authors
ORTHOPTERA			
Cockroach	Nervous system	Arthropod analogue of acetylcholine	Prosser (1952)
LEPIDOPTERA			
Arctia caja	Prothoracic defensive gland	β,β-Dimethyl acrylylcholine	Bisset *et al.* (1960)
	Abdomen	Chemically similar to acetylcholine	Gill *et al.* (1961)
HYMENOPTERA			
Apis mellifera	Heads	Two unknown compounds	Augustinsson and Grahn (1954)

confirmed by Colhoun and Spencer (1959 and unpublished data). The chemical and pharmacological evidence of Mehrotra (1960a) was conclusive evidence that developing embryos of the milkweed bug and housefly contained ACh. Despite these findings only a few species have been thoroughly tested to ensure that a pharmacologically active substance is ACh. Furthermore much of the evidence for the occurrence of ACh has been obtained with parts of bodies and not with individual organs or tissues. Results obtained in this manner may be complicated by the presence of another ester with pharmacological activity.

Information shown in Table II indicates that insects contain at least another choline ester identified as β,β-dimethylacrylycholine (Bisset *et al.*, 1960). The substance, almost as active as ACh when tested upon the guinea pig ileum, was found in the prothoracic defensive gland of the

garden tiger moth, *Arctia caja*. Gill *et al.* (1961) believe that the abdomen of the same species contains a substance chemically like ACh but differing in biological activity when tested upon a number of pharmacological preparations. It is evident that the latter workers have difficulty in reconciling chemical and biological data, but their results may show that another highly pharmacologically active choline ester has been found in an insect. The statement of Prosser (1952) appears to have been corrected by the results of Chang and Kearns (1955). The question remains whether the heads of bees contain more than one choline ester. Chefurka and Smallman (1956) pointed to difficulties in making chromatograms of extracts of insect tissues. The anionic form of ACh and concentrations of extracts led to artifacts on chromatograms. Perhaps Augustinsson and Grahn (1954) unwittingly experienced similar results. However, a reinvestigation of choline esters in heads of bees would seem to be a worthwhile project.

The identification and occurrence of choline esters in insects can be reduced to ACh and β,β-dimethylacrylylcholine, the latter being found in an external gland of a lepidopterous insect. Although feasible the occurrence of another choline ester in insects to equal the pharmacological activity of ACh has not been proven.

B. DISTRIBUTION OF ACETYLCHOLINE

To discuss the distribution of ACh in insects an assumption is made that data given in Table I are authentic although not unequivocal. A further difficulty is evident when comparing results of various workers for the same species of insect. It would seem profitable to first digress briefly and account for discrepancies in amounts of ACh obtained from insects.

Lewis and Smallman (1956) carried out experiments to determine a satisfactory method of extracting ACh from insects. Subsequently a series of papers appeared (Colhoun, 1958b; Fowler and Lewis, 1958; Colhoun and Gilleberg, 1961) showing advantageous procedures for the extraction and pharmacological assay of ACh. The results obtained by the various workers would tend to indicate that results given in Table I to about 1956 are probably unreliable. In the extraction of ACh from tissues attention must be paid to loss caused by either enzymic or non-enzymic hydrolysis. Even in frozen tissue ACh was shown to be destroyed during the process of grinding or homogenization due to rapid hydrolysis by AChE during thawing of the tissue. Lewis and Smallman (1956) found that placing of tissues in a boiling medium at

pH 3·9–4·0 (the stable pH of ACh at all temperatures) gave the most consistent and highest value of ACh. Fowler and Lewis (1958), who investigated freezing methods for extraction of ACh, confirmed this finding. Boiling therefore accomplishes two purposes: (*a*) inactivation of AChE, (*b*) denaturation of other proteins. In the method of Lewis and Smallman homogenization of tissue in a solution of trichloroacetic acid further promoted deproteinization, and careful centrifugation procedures yielded a clear extract containing ACh. Use of trichloroacetic acid requires neutralization of the extract before pharmacological assay. The experiments of Colhoun (1958a) indicated that during addition of NaOH for neutralization of the extract rapid stirring was necessary. In experiments with nervous tissue Colhoun and Gilleberg (1961) preferred the use of HCl to trichloroacetic acid as extracts treated with the organic acid were found to have a depressant effect upon the rectus abdominus muscle of the frog.

Another source of error would appear to be the improper use of extracts during pharmacological assay. Feldberg (1945) showed that erroneous results were obtained with the frog rectus abdominis muscle if the tissue and an aqueous sample of ACh were assayed in succession upon the muscle. The extract should be divided into two equal aliquots, one of which is treated with strong NaOH to destroy ACh (heating is not necessary). An aliquot of the untreated extract is assayed on the muscle and following washing and relaxation of the muscle an equal amount of the treated extract is used to which a known amount of chemically pure ACh has been added. Furthermore in routine assay procedures a four point assay system should be used where doubling of the aliquot of tissue extract can be matched by an equivalent increase in the amount of ACh added to the destroyed extract. More than one muscle assay system should be used for comparative purposes (see MacIntosh and Perry, 1950).

Colhoun (1958a) showed that temperature of acclimatization of *Periplaneta* had an effect upon the ACh content of thoracic nerve cords. Unpublished observations showed that severe treatment of the insect with CO_2 resulted in high loss of ACh. The amount of ACh in tissues during development can vary considerably (Van Der Kloot, 1955; Mehrotra, 1960a). Therefore factors influencing the physiology of insects have an important bearing upon ACh levels. A minor but probably important matter in comparison of ACh values is the choice of mathematical expression. To give results in terms of μg per g/tissue weight depends upon wet or dry weight and the necessity of weighing tissues. In certain types of physiological experiments this practice may lead to

uncertain results. A better mathematical expression of the content of ACh might be in amount of ACh per numbers of heads, ganglia, etc.

Turning to Table I, the only species of insect thoroughly investigated for its ACh content is *Periplaneta* (Colhoun, 1958a). The distribution of ACh and other elements of the cholinergic system appear to be confined to nervous tissue. The results for AChE and ChA were obtained by biochemical techniques. Low levels of enzyme activities may have escaped notice as Means (1942), by use of the Cartesian diver technique, obtained evidence of the presence of AChE in blood, heart and leg muscle of *Melanoplus differentialis*. The lowest activity of AChE in *Periplaneta* greatly exceeded the highest result obtained by Means so it is improbable that the enzyme activity would have been missed easily in a tissue of the cockroach. Good agreement was found in the distribution of ACh and ChA; enzyme concentration and amounts of ACh seemed to be correlated. In other adult insects many of the values obtained for ACh refer to heads. It is probable in these insects that the ACh titre is confined to neural elements of the head. The ACh content of eggs of insects varies considerably between species of the same and different class. As much as 8 mg per g/eggs has been found in Lepidoptera.

The distribution of ACh in non-nervous tissue of insects is of great interest. So far these results are limited to species of Lepidoptera and Hymenoptera where wide variation in ACh content is evident. Dry venom sacs of *Vespa crabro* contain 50 mg/g/ACh, whereas a secretion of a different sort and purpose, royal jelly, has about 1 mg/g. The high titre of ACh in male and female reproductive organs of Lepidoptera is unparalleled in the animal kingdom. Evidence for the occurrence of ChA and AChE in these organs would point to local hormone function.

Only a few of the total number of insect species have been subjected to a search for ACh. These experiments have revealed the occurrence of ACh in nervous tissue, non-neural elements and glandular secretions. Examinations of eggs, pupae and adults have indicated that ACh is found in all stages of insect development. Curiously very little data has been obtained for larval stages of insects. The function of ACh in insects invites investigations into the physiology of the nervous system, effects of glandular secretions upon other insects and organisms, and possible metabolic effects within tissues of non-neural origin.

III. Choline Acetylase and Acetylcholinesterase

A great deal of study on the physiological significance of ACh in insects has been directed towards their function in nervous tissue. The

purpose here has been to obtain evidence, or otherwise, of chemical transmission of nervous activity along the membranes of nerve cells or between neurones in ganglia. Certain biophysical events are associated with nervous activity, the most important being the resting and active membrane potential. In transmission of the nerve impulse at a synapse a temporal factor assumes significance in so far as the passage of a second impulse cannot normally be accomplished until the cell membrane returns to the resting condition. Relative to the temporal factor properties and intracellular distribution of the enzymes ChA and AChE are important in an overall evaluation of the events of chemical transmission of nervous activity. Indeed a study of acetylcholine in physiological function is virtually meaningless without evidence for the occurrence of ChA and AChE.

A. CHOLINE ACETYLASE

The elucidation of the probable mechanism of ACh synthesis in insects can be attributed to the clear and consise study of Smallman (1956) with heads of blowfly, although previously Tobias *et al.* (1946), Lewis (1953), and Van Der Kloot (1955) obtained evidence that insect tissues were capable of synthesizing ACh (in the instance of Van Der Kloot a cholinergic substance). In insects, as in other organisms, synthesis of ACh *in vitro* is a two-step mechanism (Smallman, 1956 and 1961).

I. ATP + CoA + acetate $\rightleftharpoons$ acetyl CoA + AMP + pyrophosphate
II. Acetyl CoA + choline $\rightarrow$ ACh + CoA

In the first reaction CoA is acetylated to acetyl CoA, while in the second step acetyl CoA provides the source of acetyl groups and energy for the enzymic acetylation of choline. ChA is the enzyme catalysing the second reaction. *In vitro* the above system, or one using phosphotransacetylase and acetyl phosphate in lieu of ATP (Colhoun, 1958c; Mehrotra, 1960a), can be used for the demonstration of ChA activity. Smallman (1956) showed that acetyl CoA formation was a limiting step in the synthesis of ACh and that the acetate utilizing system was more effective than citrate. Winteringham and Harrison (1961) demonstrated the rapid formation of (^{14}C) ACh when (2–^{14}C) acetate was injected into houseflies. However, isotopic equilibrium with the labelled acetate was never achieved. Rothschild and Howden (1961) claim that citrate is as effective as acetate as a donor system in synthesis of ACh in the housefly. Unfortunately information of this kind is absent in the report of Frontali (1958) who first studied synthesis of ACh in the

housefly. Other results of Frontali show that propionate gave rise to the corresponding ester but that phosphorylcholine was not utilized.

By conventional ammonium sulphate precipitation techniques Mehrotra (1961) purified ChA twentyfold from extracts of housefly heads. The Michaelis–Menten constant (K_m) of the enzyme for acetyl-CoA was $4{\cdot}6 \times 10^{-4}$ M. These results were compared with K_m values for the enzyme of rabbit brain and squid ganglion. Mehrotra suggests that differences in K_m for acetyl-CoA reflects an affinity for substrates. Furthermore the fact that ChA of housefly and *Periplaneta* was not inhibited by iodacetic acid (Boccacci, 1960) and that the temperature optimum of ChA of the same insects was 30°C (Colhoun, 1958c) led Mehrotra to conclude that the ChA of insects may differ in protein structure from ChA of other organisms. The differences may have great physiological importance.

B. ACETYLCHOLINESTERASE

The properties of AChE in vertebrate and invertebrate tissues have been thoroughly discussed by O'Brien (1960). In consideration of another review being prepared by Professor L. E. Chadwick, Department of Entomology, University of Illinois, no useful purpose would be served at the present time in an overall discussion of the properties of AChE. Relative to the function of ACh it is necessary to establish that the enzyme is strategically located in tissue for the enzymic destruction of the ester and that the rate of hydrolysis could be accomplished within a temporal factor (Yamasaki and Narahashi, 1960) of transynaptic conduction. Table I summarizes the rates of hydrolysis of ACh by AChE from various species of insects and shows that AChE has the potential to account for ACh liberated *in vivo*. In fact, the turnover rate of insect AChE on a tissue weight basis exceeds that found in vertebrates (see Smallman, 1958, for comparative values). Dauterman *et al.* (1962) have partially purified AChE from housefly heads. Their results indicate that the turnover number is in the neighbourhood of 110,000 with a possible molecular weight of 3 to 4 million. Furthermore, the evidence would seem to show that flyhead AChE is distinctly different from bovine erythrocyte AChE but despite this a "true" AChE. No evidence was found of a second AChE and it is doubtful that a "pseudo" AChE is present in the housefly. The hydrolytic potential of AChE of the nervous tissue of *Periplaneta* may be greater than that suspected at the moment, for Colhoun (1961) showed that inclusion of small amounts of organic solvents in the Warburg reaction

mixture increased the rate of hydrolysis of ACh nearly fourfold. A similar effect was found after solubilization of the enzyme by treatment with sodium cholate. A proper interpretation of these results is not clear at the moment but it is possible that the environment of the enzyme has a controlling influence on the rate of hydrolysis of substrate. Thus purification of the enzyme and a study of the reaction mechanism would seem to be imperative in an understanding of AChE of cockroach nervous tissue.

The distribution of AChE in insect tissues has been sought by biochemical and histochemical techniques. The latter method has yielded an insight into intracellular distribution not obtainable by biochemical determinations of enzyme activity. So far histochemical distribution of AChE has been obtained for *Rhodnius prolixus* (Wigglesworth, 1958) and *Periplaneta* (Iyatomi and Kanehisa, 1958; Kanehisa, 1961; Hamori, 1961). The most detailed study on the distribution of esterases in an insect was reported by Wigglesworth (*loc. cit.*). In the central nervous system true AChE was located in the neuropile, particularly in regions where the interneuronal material was most abundant. The probable sites of AChE in the neuropile are described by Wigglesworth, who writes: "In these places (as studied with the electron microscope) the axons and dendrites are exceedingly fine and the material between them is filled with double membranes (endoplasmic reticulum) which are largely responsible for the deep staining with osmium and ethyl gallate. This cytoplasm, which also contains mitochondria, appears to be the product of the neuroglial cells and it seems likely that this is the site of cholinesterase! In peripheral nerves AChE was found in interaxonal material, the product of neuroglial cells." Significantly, no positive evidence of AChE was found in axon contents. Kanehisa (1958), who looked for AChE in *Periplaneta*, found that the enzyme was located on nerve sheaths and the surface of neurones and that the topography of the latter could be determined by esterase distribution. Although the terms "nerve sheath" and "surface of neurones" indicates little about the fine structure of nerve fibres in *Periplaneta* it is likely that the location of AChE in this insect is similar to that in Rhodnius (for morphological fine structure see Hess, 1958; Wigglesworth, 1960). Hamori (1961), concentrating upon the localization of AChE in motor axons and their terminal arborizations in various types of muscle synapsis, concluded that although high amounts of an AChE-like enzyme were concentrated in the arborizations, that it was improbable that the enzyme was involved in neuromuscular transmission. This result agrees with the firm statement of Wigglesworth (1958) that the

muscle end plates of *Rhodnius* are not the site of any esterase. Thus the failure to detect ACh, ChA or AChE in selected muscles systems of *Periplaneta* (Colhoun, 1958a, c and 1959a) by biochemical and pharmacological techniques implies that ACh cannot be a chemical mediator of neuromuscular transmission in insects. The evidence of Colhoun points to the occurrence of elements of the cholinergic system in peripherical nerve fibres of *Periplaneta*. What then is the purpose of the system in the nerve fibres? It could be that peripheral neurones of insects are cholinergic but that the transmitter substance located at the nerve endings is of an entirely different nature.

The results of Wigglesworth (1958) and Kanehisa (1961) show that a number of esterases are found in *Rhodnius* and *Periplaneta*. In the former insect AChE was limited to neural tissue. Non-specific esterases were found in ganglionic cells, glial layer and between these cells, perineurinum cells, salivary glands, alimentary canal, pericardial cells, haemocytes, oenocytes, dermal glands, epidermal cells, germ cells and fat body. These results are of interest in view of the non-specific distribution of ali-esterase(s) in *Periplaneta* (Colhoun, 1960a). Histochemical results of Kanehisa show that AChE activity was found in nervous tissue, muscle, digestive organs reproductive organs and blood of *Periplaneta*. The occurrence of AChE in blood of *Periplaneta* was not found by Colhoun (1959a). The location of AChE in muscle, digestive and reproductive organs has not been clarified by Kanehisa. Does it perhaps refer to the nerve fibres innervating these tissues? In nervous tissue ali-esterase had about the same distribution as AChE while lipase was found in digestive organs.

Effects of inhibitors of AChE have been determined by histochemical technique (Winton *et al.*, 1958; Wigglesworth, 1958; Kanehisa, 1961). The clear-cut results obtained by Wigglesworth for *Rhodnius* show a difference in anticholinesterase properties of several compounds. Eserine, iso-OMPA and 62.C.47 inhibited only AChE but paraoxon, although inhibiting the same enzyme, partially inhibited non-specific esterases. In a study of the role of esterases in organophosphorus intoxication in the housefly (Stegwee, 1960) eserine was used as a selective inhibitor of AChE. The *in vivo* results of Wigglesworth are in accord with the *in vitro* findings of Stegwee.

IV. Acetylcholine in Physiological Systems of Insects

So far a discussion of the physiological significance of ACh in insects has been avoided. Specifically, a proven function of ACh in any species

of insects has yet to be determined. At the moment we are concerned with evidence implicating ACh in physiological function. Let us deal with this in an orderly fashion in various morphological stages of insects.

A. DEVELOPMENT

1. *Eggs of insects*

Tahmisian (1943) detected AChE in developing eggs of *Melanoplus differentialis*. Over a period of 5 years the work of Yushima and Chino revealed the presence of all elements of the cholinergic system in eggs of Lepidoptera. Most of the results have been tabulated in Table I. Their work (Yushima and Chino, 1953; Chino and Yushima, 1953; Chino and Yushima, 1954; Yushima, 1956a, b; Yushima, 1957; Chino, 1957; Yushima, 1958; Yushima and Chino, 1958), valuable in pointing to the occurrence of elements of the cholinergic system in developing embryos, suffers in clarity by use of poor pharmacological technique for assay of the substance thought to be ACh. Thus fluctuation in curves of ACh during the period of embryonic development may be due to interfering factors. No particular criticism is implied other than a difficulty in extrapolating their data. Indeed, results of these workers suggest that elements of the cholinergic system are associated with the nervous system of developing embryos. It would appear that not all eggs of species of Lepidoptera examined contained ACh. Perhaps this is due to the stage of embryonic development.

The most complete study of the cholinergic system in developing insect eggs was carried out by Mehrotra (1960a) for eggs of the milkweed bug, *Oncopeltus fasciatus* and housefly. An interesting feature of this work was the choice of two disimilar species of insect, the milkweed bug requiring at least 5 days for embryonic development and the housefly about 11 h. Contemporary and careful techniques were used in the identification of ACh and the activities of ChA and AChE. In eggs of the housefly ChA was detected at 5 h and enzyme activity increased until just prior to hatching. AChE activity was first observed at 7 h, and ACh at 9 h. Prior to hatching the ACh content was about 462 μg per g/eggs. Thus between 9 and 11 h of development the ACh content increased enormously. ChA was detected in *Oncopeltus* at 2 days while AChE and ChA appeared simultaneously at 4 days. The highest amount of ACh in eggs of this insect was 201 μg per g/eggs which at 5 days was about half of that found in eggs of the housefly. No evidence is available about the relative amounts of AChA, ChA or AChE in larvae or nymphs following hatching. A significant fall in the

ACh content of newly hatched larvae or nymphs would have suggested a shift in the dynamics of the cholinergic system and perhaps the time at which the nervous system integrated function.

Mehrotra compared results for the appearance of the cholinergic system in the two species of eggs with the embryological studies of Formiggoni (1954) for housefly, and of Butt (1949) for *Oncopeltus*. The comparison revealed that detection of ChA coincided with the appearance of neuroplasts. If neuroplasts of the developing nervous system are the source of ChA then there is an association between the nervous tissue of embryos and the cholinergic system.

Inhibition of AChE of insect eggs may be of importance in the ovicidal action of organophosphorus compounds (Smith and Wagenknecht, 1958). Apart from indicating a possible lesion of organophosphate intoxication these poisons have been of use in determining significance of the cholinergic system in developing embryos. This possibility was realized by Mehrotra and Smallman (1957) and Mehrotra (1960b) who studied the effects of exposure to parathion upon eggs of the housefly and milkweed bug during embryonic development. A previous study (Mehrotra, 1960a) had shown the occurrence of elements of the cholinergic system, so firm data was on hand for comparative work with an inhibitor of AChE.

The results showed that parathion (presumably paraoxon, following oxidation of parathion *in vivo*) inhibited AChE of the eggs and that the ACh content of the eggs increased over the amount found in untreated embryos. However, embryonic development continued to the hatching stage. At this point evidence of toxicity was obtained as the fully developed larvae failed to hatch. Similar results were found by David (1959) who studied the ovicidal action of paraoxon upon eggs of *Pieris brassicae*. The inhibition of AChE and a high increase in the ACh content found just before hatching led David to conclude that it was ACh that prevented the eggs from hatching.

The evidence from three disimilar species of insect eggs suggests that it is interference with ACh and AChE, resulting in failure of the nervous system, that leads to the eventual death of the fully developed embryo. The fact that embryonic development continued during inhibition of AChE and accumulation of ACh, indicates that AChE was not essential to growth and that ACh did not disrupt activities of cells concerned with embryogenesis. Failure of eggs to hatch suggests that mature embryos are incapable of performing the necessary movements to escape from eggs. Comparative data on the ACh titre and nervous activity of eggs beyond the hatching point would be of importance in

explaining this possibility. On the other hand accumulation of ACh could represent stimulation of synthesis of ACh *in vivo* by an effect of the poison upon some component of the synthesis mechanism. The accumulation of ACh would be of little consequence providing the ester was held in the cell in a non-toxic form. The physiological lesion of intoxication would only be evident at hatching when inhibition of AChE would prevent normal interplay of nervous activity. This explanation does not agree *in toto* with results of Potter *et al.* (1957) who studied the embryology of *Pieris* after treatment with TEPP. Heavy doses of TEPP arrested development of embryos in an early stage while with decreasing dosages development continued to hatching. In consideration that a heavy dose of TEPP meant a 6 to 12% concentration, perhaps it is not surprising that development of the embryo was prevented. However, the possibility that TEPP causes other lesions of poisoning, apart from AChE inhibition, cannot be precluded on the basis of concentration alone.

3. *Diapause*

An extremely important piece of evidence about AChE and electrical nervous activity was found by Van Der Kloot (1955) who showed that diapause in brains of *Platysamia cecropia* was accompanied by electrical silence and the absence of AChE. In contrast the abdominal nerve cord exhibited nervous activity throughout the period of diapause. Examination of brains showed the presence of a cholinergic substance which increased in titre during diapause. Evidence of a synthesis mechanism for the cholinergic substance was also detected. At cessation of diapause AChE and electrical activity returned to the brain and at this point the cholinergic substance decreased. Thus it would seem that the system had become functional again. There is some evidence that, in vertebrates, during sleep or under anaesthesia the content of ACh in brain increases until consciousness returns, when the titre falls to the normal level. Experiments carried out with diapausing brains of *Telea polyphemus* and *Sami walkerii* showed the same results found with Cecropia, whereas in brain of a non-diapausing insect both electrical activity and AChE were easily detected. Van Der Kloot suggested that the re-appearance of AChE was elicited by high substrate concentrations (the cholinergic substance accumulating during diapause) thus when AChE reappeared the brain became electrically active enabling neurosecretory cells to release hormone and ending diapause. Proof of this mechanism would show the importance of the cholinergic system in the function of the insect brain and that hormone release is under nervous control.

The possibility of induction of AChE activity by substrate is not altogether far-fetched. Goldstein (1959) showed that AChE could be synthesized in a bacterium treated with either choline or choline esters. Choline was more effective than the esters.

The results of Van Der Kloot show indirectly that accumulation of the cholinergic substance was not toxic (reminiscent of ACh in insect eggs during embryogenesis). The question thus arises concerning the form (whether bound or free in the cytoplasm) and the cytological location of the cholinergic active substance in neurones of the brain. If the substance was synthesized into a bound form within the cell what stimulus would be required to release it so that synthesis of AChE could take place? The results of Wigglesworth (1958) show that AChE in nervous tissue is predominately localized in cytoplasm of neuroglial cells. Thus the occurrence and synthesis of ACh in axoplasm would suggest a spatial difference in the location of ACh and AChE in nervous tissue. A bound form of ACh in diapausing brain of Cecropia would eliminate it as a means of causing synthesis of AChE unless the ester was freed from the bound form and able to diffuse to the site of synthesis of AChE. It would be desirable to know something about the intracellular distribution of AChE, ChA and ACh in brain of Cecropia.

A curious feature of the results obtained by Van Der Kloot is the apparent sharp division in electrical activity of diapausing brain and that of the ventral nerve cord. Surely neurones of the ventral nerve cord have terminal aborizations in brain and, conversely, neurones having their origin in brain would have processes extending into the ventral nerve cord. A theoretical consequence of this would be some electrical activity in brain and partial absence of electrical activity in the abdominal nerve cord.

Electrical nervous activity is recorded by measuring action potentials of nerve cells. In diapausing electrically silent brains action potentials are absent. Since action potentials are caused by momentary fluxes of ions (K^+ and Na^+) responsible for the resting membrane potential, a logical query would be whether the resting membrane potential is absent in diapausing brains. If so, then a fundamental difference will be found between neurones of the ventral nerve cord and those of brain. Could a nerve cell remain viable in the absence of the resting membrane potential? Do metabolic processes underlie the resting membrane potential? Indeed, use of the brain of Cecropia may provide answers to the fundamental problems of excitation of nerve cells. Perhaps resolution of the purpose of AChE in axonic conduction.

Monro (1958) attempted to link AChE with neurosecretion in brains

of three species of Lepidoptera. Although some data were obtained by use of eserine, as an inhibitor of AChE, the results are questionable as Monro failed to measure AChE activity at any time during the course of the experiment. In fact, no evidence was obtained that the brains contained AChE.

B. ADULT INSECTS

1. *Externally applied ACh*

A problem to be disposed of at the outset is the failure of ACh applied directly to nervous tissue to abolish nervous transmission (Roeder, 1948) and the inability of ACh injected into insects to cause toxicity (Hopf, 1952). The latter result is reminescent of the inability of ACh at physiological concentrations to penetrate the brain blood barrier of vertebrates. Twarog and Roeder (1957) provided a partial answer to the problem by teasing away the connective tissue sheath enveloping the sixth abdominal ganglion of *Periplaneta*. Perfusion of the ganglion with saline containing ACh caused transynaptic block. The concentration of ACh was less than amounts shown ineffective when applied to intact ganglia. However, the authors suggested that penetration of ACh to synaptic sites might also be retarded by other barriers within the ganglion.

O'Brien (1957) attacked the problem from a different point of view by considering the ionization of ACh at physiological pH. By use of a number of other compounds (O'Brien and Fisher, 1958) the evidence showed the likelihood that insects have an "ion-barrier". The whereabouts of such a barrier is of interest. It could, for example, be a function of the connective tissue sheath of the nervous system or of the mitochondrial rich, convoluted membrane layer underlying the inner edge of the sheath (see microphotograph in O'Brien, 1960). Perhaps active control of ion movement occurs in this region (but see Smith and Treherne, 1963).

Would the presence of an "ion-barrier" completely explain the lack of toxicity of ACh injected into insects? In vertebrates the motor endplate of skeletal muscle is exceedingly sensitive to ACh, but in *Periplaneta* and *Rhodnius* evidence has been discussed previously in the text that elements of the cholinergic system are absent in muscle. Thus we have a fundamental difference between the neuromuscular apparatus of vertebrates and that of two species of insect and a possible explanation for lack of peripheral effects of injected ACh. The result also indicates that membranes of neuromuscular junctions have specific receptor sites for ACh and the membranes *per se* are not necessarily stimulated. If this explanation is valid then we are left with a consideration of effects

of ACh upon organs of digestion, excretion and circulation. Ten Cate (1924) showed that ACh stimulated contractions of the foregut of *Dytiscus marginalis* while Krigsman *et al.* (1950) and Unger (1957) found that ACh affected the rate of heart beat of *Periplaneta*. Would disruption of function of these organs cause the insect to die? Here, again, there may be a point of departure between insects and mammals, for temporary interference with heart and gut may be of no profound consequence to the insect.

2. *ACh in central nervous tissue*

The ingenuous and far-thinking experiments of Mikalonis and Brown (1941) suggested an association between nervous activity of isolated nerve cords, inhibition of AChE and accumulation of ACh. The results of Chang and Kearns (1955) made possible a reinvestigation of the association between nervous activity and ACh as sound evidence was obtained that nervous tissue contained only one choline ester, that being ACh. Thus Colhoun (1958b), who had determined by pharmacological techniques the tissue distribution of ACh in *Periplaneta* (Colhoun, 1958a), subjected isolated nerve cords to stimulation in the presence of eserine and analysed the ACh content. Three basic preparations were used, in two of which the thoracic portions of the nerve cord were placed in pools of saline containing eserine. These were stimulated at the posterior portion of the abdominal cord electrically or by air puffs. The latter operation was accomplished by leaving the anal cerci attached to the cercal nerves and air puffs were directed to the cerci with the apparatus designed by Miller and Colhoun (1958). The third method was by placing the sixth abdominal ganglion in a pool of eserinized saline and stimulating the cercal nerves. The thoracic portion of the nerve cord was kept in a pool of non-eserinized saline. Because of spontaneous nervous activity it was necessary to use an eserine control as well as isolated nerve cords that had not been treated with the anticholinesterase.

In the presence of eserine the ACh content of isolated nerve cords increased significantly above the amount found in normal isolated nerve cords. Following electrical or air-puff stimulation the ACh level increased above the amount detected in nerve cords exposed to eserine alone. The feasible interpretation of the results seems to be that the increase in amount of ACh in the presence of eserine was caused by inhibition of AChE and the resultant increase in spontaneous nervous activity. Additional stimulation led to a further increase in ACh. Therefore a correlation between the level of nervous activity, ACh and

inhibition of AChE. An attempt was made to obtain correlation of disruption of transmission of nerve impulses in the sixth abdominal ganglion of *Periplaneta* with an increase of ACh following stimulation of the anal cerci. The results proved negative, for compilation of results obtained with a number of preparations showed no significant increase in ACh over that in controls treated with eserine alone. Yamasaki and Narahashi (1960) investigated electrical phenomena associated with synaptic transmission in the sixth abdominal ganglion, and by use of a number of AChE inhibitors attempted to clarify the nature of the synaptic transmitter. Their data showed evidence of synaptic transmission through the ganglia with a calculated synaptic delay of 1·59 msec. Upon application of 10^{-3} *M* ACh to the desheathed ganglion a striking stimulating action was observed. ACh at concentrations of 10^{-4} *M* and 10^{-5} *M* had little effect upon transmission but in the presence of eserine, 10^{-4} *M* ACh was effective in the discharge of nervous activity. The workers questioned the authenticity of the identity of the choline ester in the sixth abdominal ganglion which was determined to be 63 μg per g/tissue (Colhoun, 1958a) and pointed out that detection of ACh in cercal nerves is indispensable to establishing the ACh theory in synapsis across the last abdominal ganglion. The identity of ACh in nervous tissue of *Periplaneta* has been established twice (Chang and Kearns, 1955; Colhoun and Spencer, 1959). It would seem unlikely that the ganglion contains another choline ester with pharmacological properties of ACh. Cercal nerves contain ACh on the basis of pharmacological observations (Colhoun, 1958a). Practical difficulties in obtaining enough nerves for chemical studies were characteristic problems in the study of ACh. Should these facts resolve the doubts expressed by Yamasaki and Narahashi it would appear possible from their results that ACh is the transmitter substance in the sixth abdominal ganglion. These workers showed that in the presence of inhibitors of AChE, that the excitatory post-synaptic potential was prolonged. To explain this it was suggested that inactivation of AChE allowed the synaptic transmitter to act upon the subsynaptic membrane for some time thus prolonging the excitatory post-synaptic potential; the synaptic transmitter must be an ester hydrolysed by AChE.

As proof of the synaptic transmitter properties of ACh under the conditions outlined above an increase in ACh must be determined, and intracellular recording electrode techniques used to determine events at the post-synaptic membrane. The presence of other pharmacologically active substances should be searched for. Furthermore it should be remembered that synaptic transmission in the sixth abdominal ganglion

of *Periplaneta* is of a special type, where a large number of cercal nerve fibres converge to synapse with relatively few larger nerve fibres (giant axons). In this manner stimulation of the anal cerci results in rapid transmission of nerve impulses to the thoracic region of the cockroach. For combined neurophysiological and pharmacological observations the frequently used sixth abdominal preparation of *Periplaneta* may not be the most desirable. Attention should be given to synapses in other ganglia, for example those controlling motor activity in the fifth leg nerve of the metathoracic ganglion. Both the nerve and ganglion are of substantial size. Indeed, it is possible that the fifth leg nerve can be divided into dorsal and ventral units consisting of bundles of motor or sensory nerve fibres. If so, it should be possible to trace origins of motor nerves in the ganglion. Investigations of descending synaptic pathways in the sixth abdominal ganglion may be profitable, as this system would involve motor fibres controlling movement of anal cerci and other organs of the posterior abdominal segment of the cockroach.

3. *Nervous activity in axons*

In vertebrates no decision, or should it be said agreement, has been reached that ACh and AChE are essential to conduction of the nerve impulse along the axon. In insects, preparations used for electrophysiological studies on the effects of anticholinesterases are of small size and it is difficult to prevent diffusion of the toxic compounds in tissue. Thus bathing of the sixth abdominal ganglion in an effluent containing eserine or TEPP would undoubtedly affect pre- and postsynaptic nerve fibres. Blockage of nervous activity in these fibres would make measurements of transynaptic conduction impossible. Roeder (1948) probably realized this and tested the effect of DFP and HETP (an impure form of TEPP) on axonic conduction in giant fibres of *Periplaneta*. The use of a high concentration of the compounds blocked conduction but, in the case of HETP, this was attributed to acidity of the substance in solution (pH 2·0–2·3). Neuralization of HETP by use of Na_2HPO_4 prevented axonic block, but DFP action was unaffected at neutral pH. The difference in action of DFP and HETP cannot be explained in terms of inhibition of AChE, for both compounds are known to inhibit the enzyme. HETP at a dilute concentration was found to block rapidly transynaptic conduction in the sixth abdominal ganglion of *Periplaneta*, thereby showing a difference in axonic conduction and the events associated with conduction of nerve impulses in the ganglion. Was this difference due to AChE inhibition?

Colhoun (1960a) investigated the role of AChE and ali-esterase(s) in conduction of nervous activity in *Periplaneta*. Pre-treatment of the cockroaches with TOCP caused complete inhibition of ali-esterase but no toxic symptoms, whereas application of TEPP to the same cockroaches resulted in complete inhibition of AChE and loss of locomotor activity culminating in paralysis and death. Transynaptic and axonic conduction were found to be unimpared in nervous tissue in which ali-esterase was inhibited. Significantly, AChE was fully inhibited in ganglia and peripheral nerve fibres (of motor and sensory origin) after treatment with TEPP. Nervous function of motor and sensory nerves appeared to be unaltered. Ganglionic transmission was cyclic and abnormal. The observations appear to show that axonic nervous activity in *Periplaneta* is independent of AChE. More systematic studies should be carried out with selected preparations, perhaps the cercal and fifth leg nerve, to determine responses under controlled levels of inhibition of AChE and electrical stimulation. The level of ACh should be determined in nerves after inhibition of AChE. Evidence of this can be found in results of Colhoun (1959b), who compared levels of ACh in thoracic ganglia and connectives of the thoracic nerve cord of *Periplaneta* following treatment with TEPP. ACh increased in both ganglia and connectives. However, these results were determined in the late stages of intoxication. The increase of ACh at this stage of poisoning may be independent of AChE inhibition.

4. *Effect of toxic compounds upon ACh*

Although the following discussion involves the use of organophosphorus and chlorinated hydrocarbon compounds, it is not the purpose here to decide the mode of action of these substances. The two types of toxic agents have the ability to increase the ACh content of insect tissues. We are concerned with reasons for this as only the organophosphorus compounds are known to inhibit AChE.

Tobias *et al.* (1946) obtained the first evidence that treatment of insects with DDT resulted in an increase in ACh. The accumulation of ACh was detected at the prostrate and subsequent stages of poisoning in thoracic nervous tissue of *Periplaneta* and for bodies of housefly, mealworm and grasshopper. No evidence for an increase in level of ACh was found in rat or frog. This piece of evidence may show a difference between vertebrates and insects in prolonged effects of intoxication. An increase of 200 % in free ACh was found in the thoracic nerve cord of the cockroach and it was concluded that the free ACh was

the toxic agent in DDT poisoning. The results also showed that AChE was not inhibited by DDT, but no reasonable explanation was offered to account for the occurrence of free ACh in nervous tissue in the presence of unhibited AChE. In view of this anomaly a comparative study was carried out (Colhoun, 1958e, 1959b) to determine the effect of DDT and TEPP upon ACh levels in nervous tissue of *Periplaneta*.

As shown in Fig. 1, treatment of *Periplaneta* with DDT and TEPP brought about an increase in the ACh content of the thoracic nerve cord. A striking difference between the ACh effect of the poisons was observed in an increase of ACh after TEPP treatment during the convulsive phase

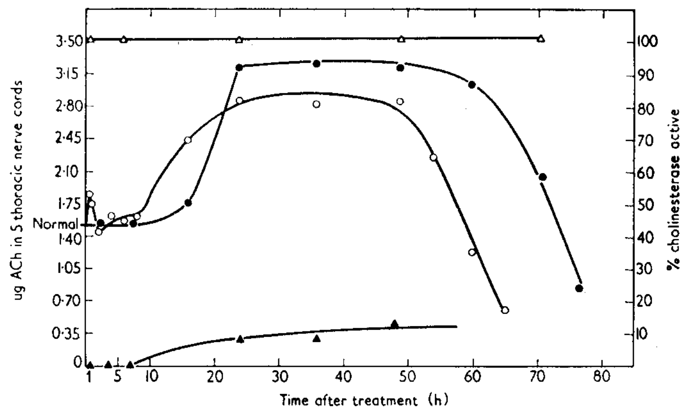


FIG. 1. Acetycholine levels and cholinesterase inhibition in roach thoracic nerve cord after treatment with TEPP and DDT. ○ ACh content after TEPP, ● ACh content after DDT, △ ChE inhibition after TEPP, ▲ ChE inhibition after DDT. Experiments for ACh levels were carried out at 20 to 22°C. (From Colhoun, 1959b.)

of intoxication. Thus in TEPP poisoning, two increases in the ACh content are apparent, the first occurring at early intoxication and the second, much higher increase, coinciding with the more paralytic stage of the insect. (The paralytic phase of poisoning is often referred to as the prostrate stage which may be defined as loss of locomotor control, the roaches being unable to walk ; they are usually found on their backs with legs and other appendages exhibiting tremors. In TEPP poisoning tremors abate within hours and a final stage of poisoning is evident some days later when tissues degenerate.) In contrast, only the second phase of ACh increase was observed in DDT poisoning; a similar result was found in dieldrin-intoxication (Colhoun, 1960b). The evidence illustrated in Fig. 1 shows that AChE was inhibited by TEPP treatment, whereas

AChE was unaffected by DDT. Lacking evidence for inteference with AChE in DDT poisoning and taking into consideration that AChE was recovering or being resynthesized in the late stage of TEPP intoxication, the detection of a high increase in ACh following treatment with the two disimilar types of poisons, suggested a common factor relative only to the paralytic condition of the cockroaches. A series of experiments were carried out to determine the *in vivo* form of ACh in nervous tissue and the distribution of ACh in ganglia and nerve fibres. The results indicated a similarity in DDT and TEPP effects upon the ACh content of nervous tissue. With these observations in mind the possible mode of action of TEPP and DDT upon ACh is as follows.

In the early stages of TEPP poisoning a rational explanation for the transitory accumulation of ACh was inhibition of AChE and greatly increased nervous transmission. At this time ACh was detected in blood of cockroaches but the origin of the released ACh was unknown. A likely reason for the increase in ACh, subsequent to the paralytic stage of DDT and TEPP poisoning, was a reduction in electrical nervous activity with a consequent rise in ACh, resulting from an "unbalance" of the normal sequence of utilization. "Unbalance" is defined in this instance as non-untilization of ACh stored in tissue because of continuing synthesis of ACh. In the normal cockroach the ACh titre remains constant, seemingly due to the dynamic equilibrium between the elements of the cholinergic system and the demands made upon it by the nervous system. At this point the results of Van Der Kloot (1955) are of interest, as the cholinergic substance increased in brain of *Cecropia* when the system was thrown out of balance.

At the paralytic phase of poisoning, attempts were made to determine the free or bound state of ACh in nervous tissue of *Periplaneta*. The evidence obtained was weighed in favour of bound ACh and, in consideration of the presence of unhibited AChE, this seemed to be the most reasonable conclusion. It is difficult to obtain comparative results for the accumulation of ACh in vertebrates following paralysis, for in these animals death has been pronounced at the cessation of respiration. If anything, the ACh titre begins to fall. Thus in insects there is the phenomenon of tissues showing metabolic function for hours, even days, after the onset of paralysis. In this way death in insects may be a matter of individual tissue degeneration. In cockroach nervous tissue the titre of ACh declined shortly before and after visible signs of necrosis. The rate of decline was such that it seemed to indicate a gradual release of ACh from the bound to the free state and hydrolysis by AChE. Tobias *et al*, (1946) showed that it was not possible to demonstrate an increase

of ACh in rats or frogs: the explanation for this may be found in the points discussed above. The examination of insect tissues beyond the early stage of intoxication may be an unnecessary complication in an elucidation of functional systems.

Lewis *et al.* (1960) and Waller and Lewis (1961) investigated the effect of a number of chlorinated hydrocarbon compounds upon the ACh content of *Periplaneta.* Their results showed, as found by previous investigators, a high increase in ACh. In addition, they showed that prostration induced by mechanical stimulation caused an increase in ACh but at lower amounts when compared with the ACh content after treatment with the chlorinated hydrocarbons. This result is significant for it indicates that the presence of a poison is not essential for an increase in ACh.

The amount of increase in ACh was different for the several types of compounds used and much higher than that previously reported. Three main conclusions are evident from their work: *a.* that ACh is a free ester in intoxication; *b.* ACh increases are found in other parts of the thorax of *Periplaneta* which cannot be accounted for by the increase in thoracic nervous tissue; *c.* in DDT poisoning the increase of ACh is detected before prostration.

Unfortunately no experimental evidence was obtained to substantiate the hypothesis of the occurrence of physiological free ACh. The occurrence of an increase in ACh before prostration may be a matter of definition differing between investigators. At just lethal concentrations DDT acts for some time before the insect becomes paralysed; therefore the onset of paralysis is more gradual than in TEPP poisoning, where convulsions and prostration is achieved rapidly. The determination of the prostrate stage in chlorinated hydrocarbon poisoning is more arbitrary; in fact, the time factor before roaches become prostrate after a lethal dose of dieldrin is about 5 days. The occurrence of high amounts of ACh in other parts of the thorax of *Periplaneta* is an important discovery. It may indicate a vast accumulation of ACh in peripheral nerve fibres which, in this insect, are known to contain ACh (Colhoun, 1958a). The reason for the increase of ACh in the fibres, should this be the location, may be found in the results of Roeder and Weiant (1951), who showed that DDT caused trains of action potentials in sensory nerve fibres of *Periplaneta.* Should this relation be proven, then a function of ACh in sensory nerve fibres is evident. The higher level of ACh in the fibres may reflect greater demands upon it by volleys of nerve impulses. Inhibition of AChE not detected *in vitro* in chlorinated hydrocarbon poisoning, would not be a requisit in this hypothesis.

Anomalies in the increase of ACh, following treatment of houseflies with a variety of organophosphorous compounds, are evident in the work of Smallman and Fisher (1958). Parathion, malathion and TEPP at lethal doses caused increases in ACh. The increases in ACh varied with the toxic substance used, and in part this may be due to the rapidity of inhibition of AChE, which in the instance of malathion (both parathion and malathion must be oxidized to paraoxon and malaoxon to become potent anticholinesterases) required a longer time than that for TEPP. At 12 hours the difference in accumulation of ACh caused by TEPP and parathion was 180%. AChE was found to be inhibited within 1 h after treatment of flies with either poison. DFP casued an initial increase in ACh which decreased within 1 h. A similar result had been reported by Winteringham and Harrison (1956), Winteringham *et al.* (1957) and Lewis and Fowler (1956). The loss of ACh with DFP, unique for the types of organophosphorus compounds used, was accounted for by redistribution of ACh through the body of the housefly. The unusual result with DFP recalls the finding of Roeder (1948) for block of axonic conduction with a high concentration of the same compound. Perhaps DFP has diverse effects upon nerve cells, one of which is inhibition of AChE. Another effect might be found in interference with permeability properties of membranes so that ACh released as a result of nervous activity diffuses away from the site of release.

In general, the results of Smallman and Fisher show that accumulations of ACh are found following inhibition of AChE. The results have been made more significant by precise determinations of AChE inhibition (Stegwee, 1960). Should increase of ACh be a measure of nervous activity in the early stage of intoxication, then an important piece of evidence has yet to be obtained. Differences in amounts of ACh at later stages of intoxication may be accounted for by a fuller understanding of the total effect of organophosphorus compounds in the housefly. Thus there may be an analogy between the housefly and the cockroach at late intoxication.

In summary, no clear-cut picture emerges from the use of toxic compounds in an evaluation of physiological significance of ACh in insects. The results are undoubtedly clouded by lack of knowledge of overall effects of poisons upon insect systems. The impact of organophosphorous compounds upon functional elements of the cholinergic system may be most evident in the early stages of intoxication. This possibility will only have significance when the purpose of ACh in nervous tissue is proven: the fundamental problem is proof of the latter.

5. *Transmission of nervous activity at neuromuscular junctions*

Elsewhere in the text evidence has been given that the cholinergic system appeared to be absent at neuromuscular junctions of insects. We can, therefore, neatly explain why ACh, curare, atropine and anticholinesterases do not block neuromuscular transmission (Roeder, 1948; Harlow, 1958; Colhoun, 1958a; Hill and Usherwood, 1961). We can also conclude that these substances are probably only effective if a specific receptor site is present at the neuromuscular junction. If we assume that neuromuscular activity in insects is mediated by a chemical compound, we are left with the certain knowledge that nothing is known about the identity of the substance or, for that matter, little about the class of compound to which it might belong. A feature of innervation of insect muscles is the finding that each muscle fibre has a nerve ending. Thus spread of surface depolarization from one fibre to another would not be necessary as in the case of vertebrate skeletal muscle where a single nerve ending innervates a group of muscle fibres. This difference between insects and vertebrates may provide a clue about the mechanism of neuromuscular transmission in insects.

Recent results of Hill and Usherwood (1961) point to inhibitory effects of tryptamine derivatives on neuromuscular transmission in the locust. High concentrations of 5-hydroxytryptamine and derivatives were necessary to affect transmission but, according to the authors, this may be due to ionization of the compounds and their inability to penetrate membranes covering the insect neuromuscular junction. They suggest that they act by blocking the receptor sites for a transmitter with a chemical structure just dissimilar enough to prevent the 5-hydroxytryptamine-like compounds substituting for it in excitation. A feature of their results was the finding that bromolysergic acid diethylamide inhibited neuromuscular transmission in the same way as 5-hydroxytryptamine. In vertebrate smooth muscle, bromolysergic acid diethylamide is an antagonist of 5-hydroxytryptamine and other indole compounds, including 5,6-dihydroxytryptamine (Colhoun and Blaschko, unpublished data).

Whether insect muscles contain an indole compound with excitatory properties remains to be solved, but the evidence of Gersch *et al.* (1961) and Colhoun (1962a) shows that nervous tissue of *Periplaneta* contains an indolealkylamine with characteristics of 5-hydroxytryptamine. The mechanism of synthesis of the compounds has been shown (Colhoun, *loc. cit.*). Are we to assume that muscle contains its own derivative of 5-hydroxytryptamine?

In a search for a chemical activator at insect neuromuscular junctions,

heed should be paid to the results of Van Der Kloot (1960) who extracted a substance from Crustacean muscles, termed Factor S, which stimulated crayfish muscle in low concentrations. Furthermore, following stimulation of motor nerves the substance was released into the muscle perfusate. The substance is thought to be a 1-substituted derivative of nicotinamide. Van Der Kloot indicates that "Catechol-4" discovered by Ostlund (1954) might be identical with Factor S. "Catechol-4" has been found in several species of insect (Ostlund, *loc. cit.*).

V. Non-Neural Function of Acetylcholine

The occurrence of acetylcholine in venom of wasp, royal jelly and honey of bees and in non-neural tissues of Lepidoptera (Table I), provides evidence of function of ACh remote from events of central nervous activity. Curiously, venom of Hymenoptera has also been found to contain histamine, 5-hydroxytryptamine and a kinin, (Jacques and Schachter, 1954; Schachter and Thain, 1954); indeed a virtual arsenal of potent pharmacologically active substances. Apart from the effect upon mammals when stung by a wasp, there is the possibility that the sting apparatus of Hymenoptera has usefulness in obtaining food, for some species prey upon insects. Also in this class of insect there are many species parasitic upon larval and pupal stages of other insects. An examination of different groups of Hymenoptera and their abdominal structures might suggest a function for the pharmacologically active substances. The susceptibility of prey paralysed by stinging may indicate a difference in physiology from species tolerating injections of high concentrations of ACh and other substances.

The high amount of ACh in royal jelly and the lesser quantity in honey may have significance in metabolism or ingestion, for both these substances are food of bees, the former being given to larval forms and the latter providing sustenance for adults during the winter. Henschler and Rhein (1960) analysed the ACh content of food eaten by polymorphic forms of bees. No firm conclusion was reached about the reason for ACh in the food. In vertebrates ACh appears to have effects upon metabolism. Hokin and Hokin (1955) obtained evidence that ACh influenced phospholipid metabolism while Pastan *et al.* (1961) found that the ester *in vitro* stimulated glucose oxidation of thyroid tissue.

The presence of ACh in reproductive organs of Lepidoptera may be a reflection of the occurrence of ChA and ACh in placenta of humans where Bull *et al.* (1961) suggested that ACh may control diffusion of ions. A search for ACh in reproductive systems of other insect species

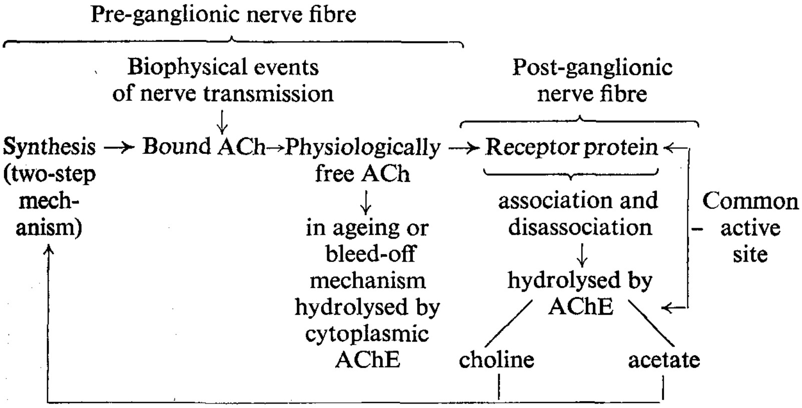
should be made to determine whether Lepidoptera are unique in this respect.

The finding of β-dimethylacrylylcholine in the defensive prothoracic gland of *Arctia caja* may be evidence (Bisset *et al.*, 1960) of a warning system in Lepidoptera, concerning which much has been written about mimicry and other phenomena of defence. The ester is almost as potent as ACh when tested upon the guinea-pig ileum and, coupled with the acrid odour associated with the compound, an effect upon sense organs mouth and digestive tract of a prospective predator (bird or insect-eating mammal) may be all that is required to discourage choice of the insect as a palatable source of food.

So far ChA and AChE has not been connected with ACh of glands, royal jelly or non-neural tissue. Colhoun and Smith (1960) attempted to locate ChA in post-cerebral and hypopharyngeal glands of bees to deduce the source of ACh in royal jelly. The results proved negative, despite the use of substrates and cofactors giving excellent results for the synthesis of ACh in extracts of nervous tissue of *Periplaneta* (Colhoun, 1958c). Detection of ChA would seem to be important in locating the source of ACh. Likewise the presence of AChE would point to the significance of ACh in intracellular or extracellular function.

VI. THE INTRACELLULAR FORM OF ACETYLCHOLINE IN INSECTS

Occasionally in the text mention has been made of bound or free ACh in nervous tissue of *Periplaneta*. These terms are loose definitions of the manner in which ACh may be present in cells. Should ACh be the transmitter of synaptic transmission, then free ACh would mean that it is available to act upon the post-synaptic membrane. In the presence of inhibited AChE the free ACh would persist at the membrane, causing disruption of synaptic transmission. On the other hand, bound ACh refers to the storage of ACh within the pre-synaptic nerve ending and in this form it cannot exert a physiological effect. When the nerve impulse arrives at the pre-synaptic ending, ACh is released to act momentarily upon the post-synaptic membrane (association) and disassociates to be hydrolysed by AChE. The hydrolysis products, choline and acetate, would be available for further synthesis of ACh. By envisaging a scheme such as this, it is apparent that complex mechanisms are at work in the cell to fit the various components of the cholinergic within the spatial and temporal factors of synaptic transmission. Aspects of this system have been lucidly described by MacIntosh (1959) and a scheme is given below to summarize probable events in vertebrates.



A method of proving the intracellular storage form of ACh has been the use of sucrose density gradients and ultra-centrifuge fractionation procedures. Whittaker (1961) described a series of experiments which tend to show that ACh and ChA of vertebrate brain are found together in synaptic vesicles. *In vivo* the vesicles are found in nerve endings. Proof of association of ACh and ChA with synaptic vesicles would pave the way to an understanding of the manner in which the pre-synaptic nerve impulse releases ACh.

Precise evidence for the storage of ACh in insect nervous tissue is yet to come, but preliminary experiments (Colhoun, 1958d) showed that ACh was in a bound form in nervous tissue of *Periplaneta*. The evidence was based upon release of ACh from homogenates of nerve cords subjected to mechanical disruption and a combination of this and osmotic pressures. The homogenates were then centrifuged and particulate and supernatant fractions were extracted and assayed for ACh. High-speed centrifugation was necessary to spin down the particulate form of ACh. Unpublished results show that ChA and ACh have the same distribution in supernatant and particulate fractions. Bellamy (1958) found that ACh and ChA were located in the light particle fraction of homogenates of heads of the desert locust. Bellamy also indicates that addition of choline to the light particle fraction increased the ACh content by 50%. Most of the ester was found to be soluble but the particle-bound ACh was higher than in the control incubation, although this never reached the original level. In essence these experiments tend to show that ACh in insects is in a bound form. Whether it will be possible to extract the bound form of ACh from tissue for density gradient experiments and electron microscopical identification of particles,

will depend upon the lability of the bound form, which in insects may differ from vertebrates. Hess (1958), in a study of the fine structure of the nervous system of *Periplaneta*, shows the presence of particles in the axoplasm of nerve fibres; the particles were found at the termination of fibres.

A recent preliminary study by Evans (1962) shows that a substance stimulating the heart of *Periplaneta* was found in a particulate fraction of homogenates of the corpus cardiacum gland. It may turn out that hormones of insects are in an intracellular bound form and thus similar to the storage of histamine, adrenalin, noradrenalin, 5-hydroxytryptamine and polypeptides in vertebrates.

VII. Other Pharmacologically Active Substances in Insects

Within the last few years a number of experiments indicate that insect tissues contain a variety of pharmacologically active substances. Although the purpose of the present paper is a discussion of the significance of ACh in insects, it would seem advantageous to outline the occurrence of other biogenic agents, for in vertebrates noradrenalin, 5-hydroxytryptamine and gamma aminobutyric acid control function in discrete areas of the nervous system.

A. Gamma-Aminobutyric Acid

Frontali (1961) showed the presence of glutamic acid decarboxylase in brains of bees. According to the same worker gamma aminobutyric acid was also detected in brain. Price (1961) believes that in houseflies the compound is found only in brain. High concentrations of the amino acid, when tested upon the isolated nerve cord of *Periplaneta*, did not interfere with electrical nervous activity (Colhoun, 1959b). Vereshchagin *et al.* (1961) tested beta-hydroxy-gamma-aminobutyric acid upon isolated nerve cords of caterpillars and adult *Dasychira pudibunda* and upon adult *Gastropacha quercifolia*. The reason for this was the report that this substance was more inhibitory in vertebrate central nervous tissue than gamma-aminobutyric acid. These workers found that the beta-hydroxy derivative inhibited electrical nervous activity in nerve cord of the caterpillar and adult states of *Dasychira*, but gamma-aminobutyric acid was less effective. Picrotoxin removed the effect of the latter substance. In contrast the beta-hydroxy compound did not inhibit action potentials of the nerve cord of *Gastropacha*. The authors conclude that the different

effects of beta-hydroxy-gamma-aminobutyric acid upon the two species of Lepidoptera is connected with physiological peculiarities of the species.

B. BIOGENIC AMINES

The most potent biogenic amines found in vertebrates are adrenalin, noradrenalin and 5-hydroxytryptamine. Adrenalin is found in the adrenals but in nerve noradrenalin is the functional amine. Adrenalin is a precursor of noradrenalin. 5-Hydroxytryptamine is a constituent of brain and intestinal mucosa. In brain there may be some link with behaviour or mental disease, and in intestine, peristalic movement.

A number of reports beginning with Cameron (1953) (see Wigglesworth, 1954 for additional information) indicate that catechol amines are found in insects. The substance extracted by Cameron from corpus cardiacum of *Periplaneta* and from whole *Tenebrio* would seem to be the insect equivalent of noradrenalin but differing from this substance in chemical properties. Gersch, Unger and Fischer (1957) claim to have successfully repeated Cameron's work. However, Ostlund (1954) and Von Euler (1961) have obtained evidence supporting the identification of adrenalin and noradrenalin in a number of insect species. The pharmacological results of Barton Browne *et al.* (1961a) and Barton Browne *et al.* (1961b) would seem to show that corpus cardiacum of *Periplaneta* contains adrenalin. The substance found by Cameron was active when applied to the hind gut and heart of *Periplaneta* and Unger (1957) believes that of a number of cardio-accelerators obtained from nervous tissue that adrenalin, noradrenalin and histamine are not among them. Further evidence of cardio-accelerators can be found in the work of Gersch *et al.* (1960), Davey (1961) and Ralph (1962). *In toto* the bewildering complexity of these results shows a need for systematic identification in tissues of the active substances mentioned above. A unifying thread between the investigators is the use of the insect heart as a test organ. So much for specificity of action.

Although 5-hydroxytryptamine had been found in venom (Jaques and Schachter, 1954) the first report of its occurrence in a tissue of an insect was given by Welsh and Moorhead (1960). Its presence in nervous tissue of *Periplaneta* was determined by use of chromatography, ultraviolet absorption and bioassay on the heart of *Helix pomata* (Gersch *et al.* 1961). Their results, although not conclusive as for nervous tissue, showed the presence of the same substance in corpus cardiacum gland. Colhoun (1962a) confirmed the results of Gersch *et al.* by use of spectrophotofluorimetry, chromatography and pharmacological activity,

the latter consisting of the rat fundus preparation in conjunction with bromolysergic acid diethylamide as the blocking agent. Furthermore, the same author reported the synthesis of 5-hydroxytryptamine in extracts of nervous tissue. The function of the indolealkylamine in nervous tissue or corpus cardiacum gland has yet to be determined. A local action is suspected (Colhoun, unpublished data).

Davey (1960) suggested that the utriculi majores of the reproductive system of *Periplaneta* contained an *o*-dihydroxyindolealkylamine which may be 5,6-dihydroxytryptamine (see Colhoun 1962b). Davey (1961) indicates that pericardial cells of the cockroach may secrete a similar compound. Colhoun (1962a) was unable to detect synthesis of 5-hydroxytryptamine in extracts of utriculi majores.

C. POLYPEPTIDES

The chemical properties of some compounds found in *Periplaneta* is suggestive of the occurrence of polypeptides. These substances seem to have physiological and biochemical effects in the cockroach. According to Davey (1961) the corpus cardiacum gland contains a substance with properties of a peptide or protein. It activated the heart in an indirect manner by causing the secretion of an indole compound from pericardial cells (located near the heart); the indole compound was thought to be the cardio-accelerator. In discussion of this result some doubt is felt by Davey about the effect of the adrenalin-like substance isolated by Cameron (1953). Proof of the mechanism illustrated by Davey would show a specificity of heart action controlled through release of a substance from the corpus cardiacum gland. However, Ralph (1961) has found wide distribution of a substance with properties of a polypeptide in tissues of *Periplaneta*. Should this substance be identical with the compound reported by Davey, then diverse effects might be found in *Periplaneta* following release from the corpus cardiacum gland. A difficulty in understanding Davey's result would be the stimulation of synthesis of the indole compound by the peptide or protein and its continued secretion following the initial treatment. That it is not entirely improbable has some support in the work of Steele (1961) who has shown that a substance, analagous to glucagon of vertebrates, caused the level of trehalose in blood of *Periplaneta* to greatly increase following an injection of a semi-purified extract of corpus cardiacum. The effect found by Steele (private communication) seems to be upon phosphorylase of fat body, where inactive phosphorylase b becomes active phosphorylase a with a consequent reduction in glycogen of fat body. The evidence shows

an effect of what is probably a polypeptide upon an enzyme system and, moreover, a glandular storage site of the substance in the cockroach.

The work of Schachter and Thain (1954) and Bhoola *et al.* (1961) shows the presence of kinin in insects. So far these observations have been made with venom of wasp and hornet. The function of the kinin is unknown but its occurrence in insects is of interest in view of the presence of bradykinin in vertebrates.

Conclusions

By an examination of the distribution of elements of the cholinergic system in insects there is every likelihood that ACh has function in the nervous system but not in synaptic transmission at neuromuscular junctions. In the nervous system there is a correlation between nervous activity and ACh when AChE is inhibited. However, this evidence is not proof that ACh is the substance acting as the transmitter of nerve impulses at synapsis of ganglia. The evidence for transmitter properties of ACh in autonomic ganglia of vertebrates is based upon the following four indications.

1. ACh is released following stimulation of preganglionic fibres. The release of ACh is detected in the presence of eserine. 2. ACh is effective in low concentrations. 3. Stimulation of the postganglionic fibres (antidromic stimulation) does not release ACh. 4. Atropine and curare which abolish the action of ACh do not prevent its release.

Finally, the presence together of ACh, ChA and AChE in ganglia and the disappearance of these following denervation. These principles are applicable to neuromuscular synapsis.

So far the weakness for the transmitter properties of ACh in insects rests upon failure to prove that ACh accumulates in nervous tissue as a fine ester following stimulation in the presence of anticholinesterases (in TEPP poisoning ACh was found in blood and ACh was translocated in houseflies treated with DFP), and no evidence for antidromic stimulation or the blocking effects of agents, such as curare and atropine. A very serious deficit is lack of good electrophysiological data demonstrating events occurring at synapsis. In this respect micro-electrode and micro-injection techniques would seem to be the only answer to an intimate analysis of the post-synaptic membrane and the effect of ACh upon it. All of these possibilities cannot preclude eventualities that synaptic transmission in insects is electrical and not chemical; if chemical the transmitter substance is not ACh. The latter conclusion will only have meaning when a substance with pharmacological

properties is found in insect tissues to participate in the spatial and temporal events of synaptic transmission.

There is no doubt that ACh is found in insect tissues of non-neural origin. The bulk of the information for non-neural function of ACh is derived from the occurrence of the ester in Hymenoptera and Lepidoptera. Whether ACh has a particular significance in species of these two classes of insect remains to be seen. Surveys for the occurrence of ChA and AChE would be of help in determining a function of ACh, as would broader coverage for the presence of the cholinergic system in non-neural tissues of other insects.

Different pharmacologically active substances are being detected in insects. Although some evidence is being obtained about physiological function, caution should be exercised in acceptance of the results, because in most instances the identity of the active compounds is uncertain. Furthermore, the use of insect organs to test pharmacological activity could be misleading until some specificity of response is obtained. Indeed, very little information is available about the pharmacology and physiology of muscle systems in insects such as gut, heart and malpighian tubules. A question to be resolved is whether control of function in these organs is direct through innervation or only accomplished by hormonal release from a gland such as the corpus cardiacum. A combination of local and remote hormonal control may be the ultimate answer. Some of these problems could be clarified by a search for pharmacologically active substances contained in the organs, for the occurrence of a substance in a tissue would do much to explain an effect of an extract of another tissue upon it. For example, the occurrence of 5-hydroxytryptamine in corpus cardiacum gland and the effect of this substance upon heart or gut does not imply that the gland controls function in these organs. The organs may have their own compliment of indoleamine and the effect of the extract of gland would be to mimic the substance stored in tissue. The same argument could apply to polypeptides, which in vertebrates have wide distribution. This is particularly true of substance P which has been found in brain, intestine and other tissues.

In an elucidation of the function of pharmacologically active substances in insects, the most fruitful results will be obtained by an awareness of the need to glean information on a broad front encompassing the disciplines of cytology, biochemistry, physiology and pharmacology. Thus dovetailing of the separate fragments of information will lead to a logical picture of events within the cell, where in the final analysis, physiological function occurs at the molecular level. Furthermore, we

must remember that survival of insects under natural conditions is a measure of their ability to cope with the external environment; in turn this reflects internal response. Chemical modifiers of behaviour at the cellular level would seem to be a goal worth working towards.

Finally, it may be evident to some readers that a discussion of the mode of action of insecticides, found to interfere with the cholinergic system, has been avoided purposely. This is true, for interference with the system is not necessarily the sole mode of action of the toxic compounds, which may have multiple effects within the insect. Economic necessity has warranted the use of compounds almost too dangerous to use in the laboratory. There is in being a division of approach, one of which is the study of the toxic agent and the other, the insect so treated. So are controversies born.

References

Augustinsson, K. B. and Grahn, M. (1954). *Acta physiol. scand.* **32**, 174–190. The occurrence of cholinesters in the honey bee.

Baeyer, A. Von (1867). Ueber das Neurin. *Liebigs Ann.* **142**, 322–326.

Barton Browne, L., Dobson, L. F., Hodgson, E. S. and Kiraly, J. K. (1961a). *Gen. comp. Endocrin.* **1**, 232–236. Adrenergic properties of the cockroach corpus cardiacum.

Barton Browne, L., Hodgson, E. S. and Kiraly, J. K. (1961b). *Science* **134**, 669–670. Stimulation of uterine contraction by extracts of the cockroach *Periplaneta.*

Bellamy, D. (1958). *Biochem. J.* **70**, 580–589. The structure and metabolic properties of tissue preparations from *Schistocerca gregaria* (Desert Locust).

Bhoola, K. D., Calle, J. D. and Schachter, M. (1961). *J. Physiol.* **159**, 167–182. Identification of acetylcholine 5-hydroxytryptamine, histamine and a new kinin in hornet venom (*V. crabro*).

Bisset, G. W., Frazer, J. F. D., Rothschild, M. and Schachter, M. (1960). *Proc. roy. Soc.* B, **152**, 255–262. A pharmacologically active choline ester and other substances in the garden tiger moth, *Arctia caja* (L.).

Blaschko, H., Colhoun, E. H. and Frontali, N. (1961). *J. Physiol.* **156**, 28P. Occurrence of amine oxidase in an insect, *Periplaneta americana* L.

Bull, G., Hebb, C. and Ratkovic, D. (1961). *Nature, Lond.* **190**, 1202. Choline acetylase in the human placenta at different stages of development.

Burgen, A. S. V. (1959). *Can. J. Biochem. Physiol.* **37**, 307–308. Symposium on neurosecretion. Introductory remarks.

Butt, F. H. (1949). *Mem. Cornell agric. Exp. Sta.* **283**, 1–43. Embryology of the milkweed bug, *Oncopeltus fasciatus* (Hemiptera).

Cameron, M. L. (1953). *Nature, Lond.* **172**, 349. Secretion of an orthodiphenol in the corpus cardiacum of the insect.

Chang, S. C. and Kearns, C. W. (1955). *Abstract. 3rd Meeting Entomol. Soc. Amer.* Cincinnati, Ohio.

Chefurka, W. and Smallman, B. N. (1955). *Nature, Lond.* **175**, 946. Identity of the acetylcholine-like substance in the housefly.

Chefurka, W. and Smallman, B. N. (1956). *Can. J. Biochem. Physiol.* **34**, 731–742. The occurrence of acetylcholine in the housefly, *Musca domestica* L.

Chino, H. and Yushima T. (1953). *Annot. zool. jap.* **26**, 233–237. On the occurrence of an acetylcholine-like substance in some insect eggs. 2. The change in acetylcholine-like content during embryonic development in some insect eggs.

Chino, H. and Yushima, T. (1954). *Zool. Mag., Tokyo* (Dobutsugaku Zasohi) **63**, 185–188. Studies on the appearance and change of cholinesterase activity in the eggs of some Lepidopterous insects.

Chino, H. (1957). *Annot. zool. jap.* **30**, 106–113. Enzymatic synthesis and hydrolysis of acetylcholine in the egg of the silk worm, *Bombyx mori.*

Colhoun, E. H. (1958a). *J. Insect Physiol.* **2**, 108–116. Acetylcholine in *Periplaneta americana* L. I. Acetylcholine levels in nervous tissue.

Colhoun, E. H. (1958b). *J. Insect Physiol.* **2**, 117–127. Acetylcholine in *Periplaneta americana* L. II. Acetylcholine and nervous activity.

Colhoun, E. H. (1958c). *Nature, Lond.* **182**, 1378. Distribution of choline acetylase in insect conductive tissue.

Colhoun, E. H. (1958d). *Nature, Lond.* **181**, 490. Physical release of acetylcholine from the thoracic nerve cord of *Periplaneta americana* L.

Colhoun, E. H. (1958e). *Science* **127**, 25. Tetraethyl pyrophosphate and acetylcholine in *Periplaneta americana.*

Colhoun, E. H. (1959a). *Can. J. Biochem. Physiol.* **37**, 1127–1134. Physiological events in organophosphorus poisoning.

Colhoun, E. H. (1959b). *Can. J. Biochem. Physiol.* **37**, 259–272. III. Acetylcholine in roaches treated with tetraethyl pyrophosphate and 2,2-Bis (p-chlorophenyl)-1,1,1-trichloroethane.

Colhoun, E. H. and Spencer, E. Y. (1959). *Science* **130**, 504–505. Acetylcholine effects of gamma-carbomethoxypropyltrimethyl-ammonium bromide.

Colhoun, E. H. (1960a). *Can. J. Biochem. Physiol.* **38**, 1363–1376. Acetylcholine in *Periplaneta americana* L. IV. The significance of esterase inhibition in intoxication, acetylcholine levels and nervous conduction.

Colhoun, E. H. (1960b). *Agric. Food Chem.* **8**, 252–257. Approaches to mechanisms of insecticidal action.

Colhoun, E. H. and Smith, M. V. (1960). *Nature, Lond.* **188**, 854–855. Neurohormonal properties of royal jelly.

Colhoun, E. H. (1961). *Nature, Lond.* **189**, 309–310. Activation of cockroach acetylcholinesterase by water-miscible organic solvents.

Colhoun, E. H. and Gilleberg, J. (1961). *Ann. ent. Soc. Amer.* **54**, 140–141. The estimation and extraction of acetylcholine from nervous tissue of the American Cockroach.

Colhoun, E. H. (1962a). *Experientia* (in press). The synthesis of 5-hydroxytryptamine in nervous tissue of *Periplaneta americana.*

Colhoun, E. H. (1962b) Aspects of biologically active substances in insects with particular reference to the cockroach, *Periplaneta americana.* 5th Int. Neurochem. Symposium, St. Wolfgang, Austria, June 1962 (to be published).

Comline, R. S. (1946). *J. Physiol.* **105**, 6P. Synthesis of acetylcholine by non-nervous tissue.

Corteggiani, E. and Serfaty, A. (1939). *C.R. Soc. Biol., Paris* **131**, 1124–1126. Acetylcholine et cholinesterase chez les insectes et les arachnides.

Dauterman, W. C., Talens, A. and van Asperen, K. (1962). *J. Insect Physiol.* **8**, 1–14. Partial purification and properties of flyhead cholinesterase.

Davey, K. G. (1960). *Canad. J. Zool.* **38**, 39–45. A pharmacologically active agent in the reproductive system of insects.

Davey, K. G. (1961). *Gen. comp. Endocrin.* **1**, 24–29. The mode of action of the heart accelerating factor from the corpus cardiacum of insects.

David, W. A. L. (1959). *J. Insect Physiol.* **3**, 14–27. The systemic insecticidal action of paraoxon on the eggs of *Picris brassicae* (L.).

Dettbarn, W. D. (1962). *Nature, Lond.* **194**, 1175–1176. Acetylcholinesterase activity in *Nitella.*

Dresden, D. (1949). Physiological investigations into the action of DDT. Ph.D. Thesis. University of Utrecht.

Elliott, T. R. (1905). *J. Physiol.* **32**, 401–476. The action of adrenalin.

Euler, U. S. von. (1961). *Nature, Lond.* **190**, 170–171. Occurrence of catecholeamines in Aevania and invertebrates.

Evans, J. J. T. (1962). *Science* **136**, 314–315. Insect neurosecretory material separated by differential centrifugation.

Feldberg, W. and Vartianen, A. (1935). *J. Physiol.* **83**, 103–128. Further observations on the physiology and pharmacology of a sympathetic ganglion.

Feldberg, W. (1945). *J. Physiol.* **103**, 367–402. Synthesis of acetylcholine by tissue of the central nervous system.

Feldberg, W. and Lin, R. C. Y. (1950). *J. Physiol.* **111**, 96–118. Synthesis of acetylcholine in the wall of the digestive tract.

Formiggoni, A. (1954). *Boll. Zool. agr. Bachio* **20**, 111–154. Studio sullo sirluppo embrionale di *Musca domestica* L.

Fowler, K. S. and Lewis, S. E. (1958). *J. Physiol.* **142**, 165–172. The extraction of acetylcholine from frozen insect tissue.

Frontali, N. (1958). *J. Insect Physiol.* **1**, 319-326. Acetylcholine synthesis in the housefly head.

Frontali, N. (1961). *Nature, Lond.* **191**, 178-179. Activity of glumatic acid decarboxylase in insect nerve tissue.

Gautrelet, J. (1938). *Bull. de l'Acad. de Med.* **120**, 285–291. The existence of an acetylcholine complex in the brain and in various organs.

Gersch, M., Unger H. and Fischer, F. (1957). *Z. Friedrich-Schiller Universitat, Jena,* **6**, 126–129. Die Isolierung eines Neurohormons aus dem Nervensystem von *Periplaneta americana* und einige biologische Testverfahren.

Gersch, M., Fischer, F., Unger, H. and Koch, H. (1960). *Z. Naturf.* **15**, 319–322. Die Isolierung neurohormonaler Faktoren aus dem Nervensystem der Küchenschabe *Periplaneta americana.*

Gersch, M., Fischer, F., Unger, H. and Kabitza, W. (1961). *Z. Naturf.* **16**, 351–352. Vorkommen von Serotonin im Nervensystem von *Periplaneta americana* L. (Insecta).

Gill, E. W., Parsons, J. A. and Paton, W. D. M. (1961). *J. Physiol.* **157**, 31–32P. Attempts to isolate pyruvylcholine.

Goldschmidt, S. and Burkert, H. (1955). *Hoppe-Syelers Z.* **301**, 78–89. Die Hydrolyse des cholinergeschen Honigwirkstoffe und anderer Cholinester Mittels Cholinesterasen und der Hemmung im Honig.

Goldstein, D. B. (1959). *J. Bact.* **78**, 695–702. Induction of cholinesterase biosynthesis in *Pseudomonas fluorescens.*

Hamori, J. (1961). *Bibl. Anat.* **2**, 194–206. Cholinesterase in insect muscle innervation with special reference to insecticide effects of DDT and DFP.

Harlow, P. A. (1958). *Ann. appl. Biol.* **46**, 55–73. The action of drugs on the nervous system of the locust (*Locusta migratoria*).

Henschler, D. (1956). *Hoppe-Seylers Z.* **305**, 34-41. Zur Identifizierung von Cholistern in biologischen Material, insbesondera von Acetylcholin in Bienenfuttersäften.

Henschler, D. and Rhein, von W. (1960). *Naturwissenschaften* **47**, 1–2. Änderungen des Acetylcholinegehaltes von Bienenfuttersäften in der Madenentwicklung.

Hess, A. (1958). *J. biophys. biochem. Cytol.* **4**, 731–742. The fine structure of nerve cells and fibres, neurolgia, and sheaths of the ganglion chain in the cockroach *Periplaneta americana.*

Hill, R. B. and Usherwood, P. N. R. (1961). *J. Physiol.* **157**, 393–401. The action of 5-hydroxytryptamine and related compounds in neuromuscular transmission in the locust *Schistocerca gregaria.*

Hokin, L. E. and Hokin, M. R. (1955). *Biochim. Biophys. Acta* **16**, 229–237. Effects of acetylcholine on phosphate turnover in phospholipids of brain cortex *in vitro.*

Hopf, H. S. (1952). *Ann. appl. Biol.* **39**, 193–202. Studies on the mode of action of insecticides. I. Injection experiments on the role of cholinesterase inhibition.

Iyatomi, K. and Kanehisa, K. (1958). *Jap. J. appl. Ent. Zool.* **2**, 1–10. Localization of cholinesterase in the American cockroach.

Jaques, R. and Schachter, M. (1954). *Brit. J. Pharmacol.* **9**, 53–58. The presence of histamine, 5-hydroxytryptamine and a potent, slow contracting substance in wasp venom.

Kanehisa, K. (1961). Studies on the cholinesterases in insects, especially in relation to the mode of action of organophosphorus insecticides. Bull. No. 2 Laboratory appl. Ent., Faculty of Agriculture, Nagoya University, Anjo, Japan.

Keil, W. and Kritter, B. (1935). *Biochem. Z.* **276**, 61–65. Chemistry and pharmacology of fermented foodstuffs.

Krigsman, B. J., Dresden, D. and Berger, N. E. (1950). *Bull. ent. Res.* **41**, 141. The action of rotenone and TEPP on the isolated heart of the cockroach.

Lewis, S. E. (1953). *Nature, Lond.* **172**, 1004–1005. Acetylcholine in blowflies.

Lewis, S. E. and Fowler, K. S. (1956). *Nature, Lond.* **178**, 919–920. Effect of diisopropylphosphoro fluoridate on the acetylcholine content of flies.

Lewis, S. E. and Smallman, B. N. (1956). *J. Physiol.* **134**, 241-256. The estimation of acetylcholine in insects.

Lewis, S. E., Waller, J. B. and Fowler, K. S. (1960). *J. Insect Physiol.* **4**, 128–137. The effect of DDT and of physically induced prostration on acetylcholine levels in this cockroach.

Lin, R. C. M. (1957). *Brit. J. Pharmacol.* **12**, 265–269. The distribution of acetylcholine in the Malayan Jack-Fruit Plant, *Artocarpus integra.*

Loewi, O. (1921). *Pflüg. Arch. ges. Physiol.* **189**, 239–242. Über humorak Übertragbarkeit der Herznervenwerkung.

MacIntosh, F. C. and Perry, W. L. M. (1950). *Methods in Medical Research* **3**, 78–92, Chicago. Biological estimation of acetylcholine.

MacIntosh, F. C. (1959). *Can. J. Biochem. Physiol.* **37**, 343–356. Formation storage and release of acetylcholine at nerve endings.

Marquardt, P. and Vogg, G. (1952). *Arzneimettel-Forsch* **2**, 152–155. Occurrence, properties, and chemical constitution of the cholinergic factor in honey.

Means, O. W. (1942). *J. cell. comp. Physiol.* **20,** 319–324. Cholinesterase activity of tissues of adult *Melanoplus differentialis* (Orthoptera, Acrididae).

Mehrotra, K. N. and Smallman, B. N. (1957). *Nature, Lond.* **180,** 97–98. Ovicidal action of organo-phosphorus insecticides.

Mehrotra, K. N. (1960a). *J. Insect. Physiol.* **5,** 129–142. Development of the cholinergic system in insect eggs.

Mehrotra, K. N. (1960b). *Can. J. Biochem. Physiol.* **38,** 1045–1052. Effect of an anticholinesterase on the cholinergic system in insect eggs.

Mehotra, K. N. (1961). *J. Insect Physiol.* **6,** 215–221. Properties of choline acetylase from the housefly, *Musca domestica.*

Metcalf, R. L. and March, R. B. (1949). *J. econ. Ent.* **42,** 721–728. Mode of action of parathion and its derivatives and their toxicity to insects.

Mikalonis, S. J. and Brown, R. H. (1941). *J. cell comp. Physiol.* **18,** 401-403. Acetylcholine and cholinesterase in the insect central nervous system.

Miller, D. M. and Colhoun, E. H. (1958). *Nature, Lond.* **181,** 126–127. An apparatus for air puff stimulation of insect sense organs.

Morley, J. and Schachter, M. (1961). *J. Physiol.* **157,** 1-2P. Identification of acetylcholine in the silk gland of the caterpillar of *Arctia caja* (L.).

Morley, J. and Schachter, M. (1962). *J. Physiol.* **162,** 12P. Acetylcholine in non-nervous tissue of the garden tiger (*Arctia caja*) and other moths.

Monro, J. (1958). *Aust. J. Biol. Sci.* **11,** 399–456. Cholinesterase and the secretion of the brain hormone in insects.

O'Brien, R. D. (1953). *Nature, Lond.* **172,** 162. Occurrence of Cholinesterase in *Tenebrio* and *Tribolium.*

O'Brien, R. D. (1957). *Ann. ent. Soc. Amer.* **50,** 223–229. Esterases in the semi-intact cockroach.

O'Brien, R. D. and Fisher, R. W. (1958). *J. econ. Ent.* **51,** 169–175. The relation between ionization and toxicity to insects for some neuropharmacological compounds.

O'Brien, R. D. (1960). "Toxic Phosphorus Esters". Academic Press, New York.

Ostlund, E. (1954). *Acta. physiol. scand.* **31,** (Suppl. 112). The distribution of catechol amines in lower animals and their effect on the heart.

Pastan, I., Herring, B., Johnson, P. and Field, J. B. (1961). *J. biol. Chem.* **236,** 340–342. Stimulation *in vitro* of glucose oxidation in thyroid by acetylcholine.

Potter, C., Lord, K. A., Kenten, J., Salkeld, E. H. and Holdbrook, D. U. (1957). *Ann. appl. Biol.* **45,** 301–375. Embryonic development and esterase activity of eggs of *Pieris brassicae* in relation to TEPP poisoning.

Price, G. M. (1961). *Biochem. J.* **80,** 420–428. Some aspects of amino acid metabolism in the adult housefly, *Musca domestica.*

Prosser, C. L. (1952). "Nerve Impulse". Trans. Third Conference, p. 51., Josiah Macy. Jr. Foundation, New York.

Ralph, C. L. (1961). *American Zoology* **1,** 381, abstract 109. Distribution of cardio-accelerator hormone in *Periplaneta americana.*

Ralph, C. L. (1962). *J. Insect Physiol.* **8,** 431–439. Heart accelerators and decelerators in the nervous system of *Periplaneta americana* L.

Roeder, K. D. (1948). *Johns Hopk. Hosp. Bull.* **83,** 587–599. V. The effect of anti-cholinesterases and related substances on nervous activity in the cockroach.

Roeder, K. D. and Weiant, E. A. (1951). *Ann. ent. Soc. Amer.* **44,** 372–380. The effect of concentration, temperature and washing on the time of appearance of DDT-induced trains in sensory fibres of the cockroach.

Rothschild, J. and Howden, G. F. (1961). *Nature, Lond.* **192,** 283–284. Effect of chlorinated hydrocarbon insecticides on insect choline acetylase, condensing enzyme and acetylkinase.

Schachter, M. and Thain, E. M. (1954). *Brit. J. Pharmacol.* **9,** 352–359. Chemical and pharmacological properties of the potent slow-contracting substance (kinin) in wasp venom.

Smallman, B. N. (1956). *J. Physiol.* **132,** 343–357. Mechanism of acetylcholine synthesis in the blowfly.

Smallman, B. N. (1958). *Proc. Xth Int. Congr. Ent.* **2,** 5–12. The physiological basis for the mode of action of organophosphorus insecticides.

Smallman, B. N. and Fisher, R. W. (1958). *Can. J. Biochem. Physiol.* **36,** 575–586. Effect of anticholinesterases on acetylcholine levels in insects.

Smallman, B. N. (1961). *Meth. in med. Res.*, **9,** 203–207, Chicago. Determination of choline acetylase activity.

Smith, D. S. and Treherne, J. E. (1963). *In* "Advances in Insect Physiology", **1,** (eds. Beament, J. W. L., Treherne, J. E. and Wigglesworth, V. B. Functional aspects of the organization of the insect nervous system. Academic Press, London.

Smith, E. H. and Wagenknecht, A. C. (1956). *J. econ. Ent.* **49,** 777–783. The occurrence of cholinesterase in eggs of the peach tree borer and large milkweed bug and its relation to the ovicidal action of parathion.

Smith, E. H. and Wagenknecht, A. C. (1958). *Proc. Xth Int. Congr. Ent.* **2,** 29–39. The occurrence of cholinesterase in insect eggs and its role in the ovicidal action of organophosphates.

Steele, J. E. (1961). *Nature, Lond.* **192,** 680–681. Occurrence of a hyperglycaemic factor in the corpus cardiacum of an insect.

Stegwee, D. (1960). *Can. J. Biochem. Physiol.* **38,** 1417–1429. The role of esterase inhibition in tetraethylpyrophosphate poisoning in the housefly, *Musca domestica* L.

Tahmisian, T. N. (1943). *J. exp. Zool.* **92,** 199–213. Enzymes in ontogenesis: cholinesterase in developing eggs of *Melanoplus differentialis* eggs.

Ten Cate, J. (1924). *Arch. neerl. Physiol.* **9,** 594–604. Contribution à la physiologie comparee du tubes digestif. III. Les movements rhythmiques spontanes de l'oesophage isolé et du gosier de *Dytiscus marginalis.*

Tobias, J. M., Kollros, J. J. and Savit, J. (1946). *J. cell. comp. Physiol.* **28,** 159–182. Acetylcholine and related substances in the cockroach, fly and crayfish and the effect of DDT.

Twarog, B. M. and Roeder, K. D. (1957). *Ann. ent. Soc. Amer.* **50,** 231–237. Pharmacological observations on the desheathed last abdominal ganglion of the cockroach.

Unger, H. (1957). *Biol. Zkl.* **76,** 204–225. Untersuchungen zur neurohormonalen Steuerung der Herzätigkeit bei Schaben.

Van Der Kloot, W. G. (1955). *Biol. Bull.* **109,** 276–294. The control of neurosecretion and diapause by physiological changes in the brain of the Cecropia silkworm.

Van Der Kloot, W. G. (1960). *J. Neurochem.* **5,** 245–252. Factor S—A substance which excites *Crustacean* muscle.

Vereshchagin, S. M., Sytinskii, I. A. and Tyshchenko, V. P. (1961). *Doklady Akademii*

Nauk SSSR **138,** 722–724. Effect of Beta-oxy-gamma-aminobutyric acid on the bioelectrical activity of the ganglia of an isolated neural chain of Lepidoptera.

Waller, J. B. and Lewis, S. E. (1961). *J. Insect Physiol.* **7,** 315–323. The effect of gamma-BHC and other insecticides on ACh levels in the cockroach.

Welsh, J. H. and Moorhead, M. (1960). *J. Neurochem,* **6,** 146–149. The quantitative distribution of 5-hydroxytryptamine in the invertebrates, especially in their nervous systems.

Whittaker, V. P. (1961). The subcellular localization of transmitter substance in the central nervous system. *Biochem. Pharmacol. Physiol. Symposium.* Publication Division, Pergamon Press, Oxford.

Wigglesworth, V. B. (1954). *Pubbl. Staz. zool. Napoli* **24,** 41–45. Neurosecretion and the corpus cardiacum of insects.

Wigglesworth, V. B. (1958). *Quart. J. micr. Sci.* **99,** 441–450. The distribution of esterase in the nervous system and other tissues of the insect *Rhodnius prolixus.*

Wigglesworth, V. B. (1960). *Quart. J. micro. Sci.* **101,** 381–388. Axon structure and the dictyosomes (Golgi bodies) in the neurones of the cockroach, *Periplaneta americana.*

Winteringham, F. P. W. and Harrison, A. (1956). *Nature, Lond.* **178,** 81–82. Study of anticholinesterase action in insects by a labelled pool technique.

Winteringham, F. P. W., Harrison, A., McKay, M. A. and Weatherley, A. (1957). *Biochem. J.* **66,** 49P. Biochemistry of Diisopropylphosphorofluoridate poisoning in the adult housefly.

Winteringham, F. P. W. and Harrison, A. (1961). *Biochem. J.* **78,** 22P. Incorporation of (2-^{14}C) acetate into acetylcholine of the adult housefly *in vivo* under conditions of rest, activity and insecticidal action.

Winton, M. Y., Metcalf, R. L. and Fukuto, T. R. (1958). *Ann. ent. Soc. Amer.* **51,** 436–441. The use of acetylthiocholine in the histochemical study of the action of organophosphorus insecticides.

Yamasaki, T. and Narahashi, T. (1960). *J. Insect Physiol.* **4,** 1–13. Synaptic transmission in the last abdominal ganglion of the cockroach.

Yushima, T. and Chino, H. (1953). *Annat. Zool. Jap.* **26,** 228–232. On the occurrence of an acetylcholine-like substance in some insect eggs. 1. On the muscle-stimulating substance in eggs of the rice stem borer, *Chilo simplex* and cabbage army worm, *Barathra brassicae.*

Yushima, T. (1956a). *Jap. J. appl. Zool,* **21,** 70–73. Distribution of the acetylcholine-like substance in eggs of some Lepidopterous insects.

Yushima, T. (1956b). *Jap. J. appl. Zool.* **21,** 129–131. Two kinds of the fluctuation curve of the acetylcholine-like substance found in the paddy borer, *Schoenobius incertulas,* during the embryonic development.

Yushima, T. (1957). *J. Econ. Ent.* **50,** 440–443. Changes in rate of synthesis of acetylcholine *in vitro* in eggs of the Asiatic rice borer, *Chilo suppressalis* (Wlk.) and the cabbage army worm, *Mamestra brassicae* (L.) during embryonic development.

Yushima, T. (1958). *Jap. J. appl. ent. Zool.* **1,** 38–42. Relation between occurrence of choline acetylase and appearance of synthetic activity of acetylcholine in eggs of rice stem borer, *Chilo suppressalis* and cabbage army worm, *Barathra brassicae,* during embryonic development.

Yushima, T. and Chino, H. (1958). *OYO-Kontyu* **9,** 52–58. Acetylcholine and cholinesterase in the rice stem borer, *Chilo simplex.*

APPENDIX

Subsequent to completion of this review paper Sternburg and Hewitt (1962) reported that function of AChE *in vivo* was impaired in DDT-intoxicated cockroaches, possibly by release of a substance that formed a chemical or physical barrier to ACh at active sites of AChE. Lindane-poisoning differed in this respect, but the authors pointed out that DDT may cause greatly increased afferent nervous activity, resulting in bombardment of cholinergic synapsis, while lindane is mostly a central nervous poison. Proof of interference with AChE by a substance preventing hydrolysis of ACh would be of importance in explaining the increase of ACh in chlorinated hydrocarbon poisoning, but in the instance of DDT intoxication it is not necessary to have intact afferent connections with the central nervous system, for Colhoun (1960b) has shown that ACh accumulated for hours in perfused isolated nerve cords obtained from just prostrate DDT-poisoned roaches. A similar result was found with dieldrin treatment which may be a central nervous poison in the cockroach. In any case the peripheral or afferent nervous effect of DDT cannot be explained on the basis of interference with AChE in the central nervous system, unless AChE of afferent nerve fibres is similarly impaired. A curious aspect of the work of Sternburg and Hewitt[1] (*loc. cit.*) is their reluctance to accept a function of ACh and AChE in organophosphorus poisoning but eager to implicate AChE in chlorinated hydrocarbon intoxication. Perhaps they believe that another substance is the endogenous substrate for AChE in nervous tissue of insects and that ACh is unimportant in transmission of nervous activity. Surely it is not necessary to induce intoxication to obtain evidence of a substance participating in transynaptic conduction.

[1] Sternburg, J. and Hewett, P. (1962). *J. Insect Physiol.* **8**, 643–663. *In vivo* protection of cholinesterase against inhibition by TEPP and its methyl homologue by prior treatment with DDT.

Feeding Behaviour and Nutrition in Grasshoppers and Locusts

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I. Food and Feeding

A. Food Plant Preferences

Reviewing early observations on the food of Acrididae, Uvarov (1928) noted that graminaceous feeders were characteristic of this family. Subsequent critical study has confirmed that grasses constitute the major food of several British grasshoppers (Clark, 1948; Richards and Waloff, 1954; Williams, 1954) and Old World and Australian plague locusts (Faure, 1923; Merton, 1959; Davey and Johnston, 1956; Johnston and Buxton, 1949; Chapman, 1957, 1959; Clark, 1947a, b). However, the principal native food plants of the Desert locust, *Schistocerca gregaria*, are now generally conceded to be herbaceous and shrubby plants (Brown, 1947; Ellis and Ashall, 1957), while an extensive study of the food preferences of North American grasshoppers (see Isely, 1938–46 for reviews) leaves little doubt that, far from being mainly consumers of grass, Acrididae (and for that matter Tettigoniidae) display great diversity in both the numbers and types of plant species used as food. In a survey of eighty-nine species of Acrididae Isely (1944) concluded that a minority, mostly in the subfamily Acridinae, were obligatorily graminivorous.

Some Oedipodine species were graminivorous or forbivorous (the term "forb" is used by American workers to denote any small, broad-leafed plant); others ate both forbs and grass although generally favouring the latter. The Cyrtocantharinae (Catantopinae) were primarily forbivorous, but some fed on trees, and many of this group, which includes the pest species of *Melanoplus*, readily took grasses as well as forbs. It might be thought that these preferences were trivial, but it was emphasized that many species starved to death rather than eat unusual food plants (Isely, 1944, 1946).

It may be wondered if the preferences of the more polyphagous species are capricious or have any nutritional significance. Growth and fecundity of *Melanoplus* spp. reared on various food plants differ widely (Hodge, 1933; Pfadt, 1949; Tauber *et al.*, 1945; Smith *et al.*, 1952; Barnes, 1955; Pickford, 1958), and in some cases choice tests showed that species allowing best growth were also most preferred (Smith, 1959; Pickford, 1962). Similar differences were found for *Camnulla pellucida* reared on pure stands of seventeen grasses; it was noted that good growth and high fecundity were not always correlated (Putman, unpublished paper). This suggests that combinations of plants may be conducive to optimal function throughout the life cycle. In cage experiments *Melanoplus* spp. often grew best with more than one plant species as food.

A noteworthy feature of studies with *Melanoplus* spp. is that, whereas certain native forbs were sometimes most preferred and resulted in better growth (as might be expected of predominately forbivorous species), at others cultivated grasses such as wheat were better. This suggests that non-taxonomic features may influence the choice of food plants. One may enquire what might cause grasshoppers which normally spurn native grasses to favour certain cultivated graminacae. Isely (1944) found that graminivorous species possessed mandibles more suited to the comminution of tough material than those of forbivorous or mixed feeders, and related this morphological distinction to the fact that grasses are on the whole tougher and harder than the leafy parts of forbs. However, young grass is comparatively succulent, and tender shoots were often accepted by forbivorous grasshoppers when the mature grass was not. In these cases food would seem to be selected on a nutritional basis.

Considerable evidence may be adduced to support the view that nutritional characteristics are as important as taxonomic status in the selection of food by many economically important acridids, for it has often been noted that locusts take fresh green vegetation if a choice is available. Of the grasses which form the principal food of *Nomadacris*

septemfasciata, the species taken preferentially in choice experiments were also those having the softest cuticle and highest water content (Chapman, 1957). The principal plants eaten by *Schistocerca* were species which remained green when others withered (Guichard, 1955), and in preference experiments leaf texture was thought to influence the choice of food (Roonwal, 1953). The point is made very clearly in ecological studies on the Australian locusts *Chortoicetes terminifera* and *C. cruciata*. Both are principally graminivorous and feed on whichever grasses are the greenest. In the presence of fresh grass dicotyledons were passed over, but with withered grass and green dicotyledons available, the latter were preferred (Clark, 1947a, b). Evidently a tenable case exists for supposing that the food preferences which many polyphagous pest grasshoppers undoubtedly exhibit are partly the outcome of the discrimination of nutritionally advantageous features.

B. FEEDING BEHAVIOUR

1. *Attraction and orientation to food plants*

In analysing the selection of food by phytophagous insects Dethier (1953) found it convenient to consider the stimuli involved under three categories: those which orientate the insect to the plant, those causing it to bite and those which maintain feeding.

Happenings which induce stimuli in the first category originate from a distance and, therefore, are likely to be concerned in vision or olfaction. That olfactory attraction by specific chemical substances found in food plants may play some part in orientation to food has been demonstrated with various oligophagous lepidopterous larvae (Dethier, 1937, 1941; Thorsteinson, 1953a; Watanabe, 1958) and adult weevils (Hans and Thorsteinson, 1961). However, as the distances over which attraction operated were never more than a few centimetres it has been suggested that the reactions were probably more concerned in inhibiting desertion from plants encountered by chance than in locating them from a distance (Thorsteinson, 1960). The term "aggregant" was proposed for this situation and seems equivalent to "arrestant" as defined by Dethier, *et al.* (1960).

It might be thought that many phytophagous insects, particularly larvae, could well dispense with the ability to locate food plants with precision, being placed on or close by them as eggs, or likely quickly to stumble on them in random foraging. Many grasshoppers seem to be in this well-provided situation; however, others frequent arid regions with sparse vegetation where the advantages to be gained from some means

of locating food at a distance are more evident. In spite of early reports suggesting that certain acridids located food by olfaction from considerable distance (Hunter and Claasen, 1914; Lépiney, 1930; Watson and Bratley, 1940; Volkonsky, 1942), more recent opinion has been that an attraction to food-plants, if it occurred, probably involved vision (Chapman, 1955; Kennedy, 1939). *Schistocerca* has the ability to distinguish linear patterns and is attracted by those with emphatic vertical components (Wallace, 1958). Similar visual reactions were found in certain British grasshoppers; vertical black lines were more attractive than horizontal. Given the choice of vertically or horizontally placed grass, the former was preferred, suggesting a visual basis for discriminating the grasses which are their normal food (Williams, 1954).

Williams interpreted other evidence to rule out olfaction in food-finding. *Locusta* hoppers could not distinguish grass from raffia in a choice-chamber, and ate normal amounts of grass after amputation of their antennae and palpi, the presumptive site of olfactory sensillae (Frings and Frings, 1949). Olfactometer experiments, likewise, afforded no evidence of an ability to detect grass, in two species of British grasshoppers. However, in contrast to these negative findings other studies have demonstrated olfactory responses that might have some bearing on food finding and discrimination. In tests of the attraction of chemicals for grasshopper baits, four ammonium salts, the amides of anisic and benzoic acids and pentyl acetate had a probably olfactory attraction for certain species of *Melanoplus*; eugenol, hexenoic and valeric acids were strongly repellent (Skoog, *et al.*, 1960; Barnes and McLellan, 1960). In these tests olfaction was not always distinguishable from contact stimulation and it was emphasized that even when attraction was clearly olfactory, it was never observed at distances of more than an inch. Although of interest in demonstrating an ability to discriminate simple odours, the significance of these observations in relation to foraging is no more than suggestive. Of more pertinence is the demonstration that *Romalea micropterus* and *Melanoplus mexicanus* detected the odours of various fresh or dried plants, bran, or water if thirsty, over distances of about a metre, by means of sensoria on the antennae (Slifer, 1955). In connection with thirst it has been found that certain grasshoppers which usually prefer a dry atmosphere become hygrophilic when starved (Riegert, 1959). *Romalea* sensed the strongly repellent odours of ten essential oils by receptors on the leg and perhaps on the body (Slifer, 1954, 1956), presumably an instance of the common chemical sense rather than olfaction as usually understood (Dethier and Chadwick, 1948).

The reaction of fourth instar *Schistocerca* to airborne plant odours provides persuasive evidence that olfaction might help locate distant food (Haskell, *et al.*, 1962). Hoppers usually walked downwind when a slow air current was passed through a special arena; however, when grass was placed in the airstream, the hoppers, if previously starved for at least 2 hours, turned upwind and moved towards the source of the plant odour. Moisture alone was ineffective, and the response increased with starvation or if crushed vegetation was used. Foliage of unrelated plants provided the stimulus, evidently of general distribution in green vegetation. Of several pure substances, three ammonium salts were attractive and valeric acid had a repellent action, a result similar to that for *Melanoplus*. Coumarin, a common constituent of the essential oils of many grasses and other plants, was without effect.

In discussing their findings in relation to those of previous workers these authors emphasize the importance of prior starvation to release the olfactory response and suggest that the negative results of Williams (1954) stemmed from the absence of suitable pre-experimental inanition. However, some unpublished observations of my own on the olfactory detection of food by *Schistocerca* accord with some of William's findings. On placing a small bundle of fresh grass in a cage of unfed hatchlings, most of them at once moved to the grass to feed. To find whether olfaction was involved, bundles of grass or damp cotton wool enclosed in muslin were together placed in the cage. In none of several tests was conspicuous movement of hoppers to the bundles observed, and no difference could be detected between the numbers that eventually settled on grass or cotton wool packages; this prompted the belief that the hoppers failed to smell grass. Somewhat similar tests with adults reared on artificial diet gave altogether different results. Groups of about fifty, of mixed sex, were kept without food overnight. On placing a dish of bran in their cage, many would wave their antennae, turn, and descend from their perches to jostle around the dish. Wheatgerm oil absorbed on to cellulose powder had a similar effect, and when a dish of plain cellulose powder was provided simultaneously it was ignored. Casein, dry sucrose, and B viatmins dried on to cellulose were entirely negative, although water-moistened cellulose powder seemed slightly attractive.

Taken in conjunction with the observations of Williams (1954) and Haskell *et al.* (1962) the view may be hazarded that while adult and fourth instar *Schistocerca* seem able to follow odour signals from some distance to their source, young hoppers might lack this facility. Why this should be so is not easily understood unless it is the case that

hatchlings normally emerge in locations liberally supplied with suitable vegetation immediately to hand, as detailed maps of *Schistocerca* oviposition sites indicate (Ellis and Ashall, 1957).

An interesting statement of Haskell *et al.* (1962) may have some relevance here. Apparently *Schistocerca* adults reared on artificial diet failed to recognize plants as food until force fed, and field observations are cited as evidence that food preferences tend to be fixed by what has already been eaten. As hatchlings have never fed, it may be envisaged that until they sample whatever is nearest to hand they may not learn the appropriate stimulus signalling food. It would be interesting to know how artificially reared hoppers which disdain grass would react to its odour in the wind tunnel situation.

Enough has been said to show that olfactory stimuli may influence the foraging activity of some acridids. Evidence obtained in still air suggests that the distances concerned are small; this is consistent with what is known of other insects, and such stimuli are likely to be concerned only with local orientation to plants. However, the wind tunnel experiments with *Schistocerca* indicate the possibility of olfactory attraction over long distances. Moths can follow olfactory sex stimuli at vast distances from their source even though the range of detection in still air is no more than a few yards (Karlson and Butenandt, 1959), and one might think that an analagous state of affairs could exist for odiferous stimuli from food.

2. *Phagostimulation*

Having located food, biting must occur and ingestion be maintained. There is little doubt that gustatory responses are involved in the acceptance (or rejection) of what is sampled on biting and in sustaining feeding thereafter. The factors which initially induce biting are less well understood; indeed, it is not clear whether they are usually distinct from those concerned in approach and orientation. Amongst phytophagous insects probably the best evidence that biting may require a specific stimulus is afforded by work on the silkworm; different substances present in mulberry leaves are said specifically to attract the larvae, stimulate biting, and maintain subsequent feeding (Watanabe, 1958; Hamamura, 1959; Hamamura and Naito, 1961; Hamamura *et al.*, 1961; Ito, 1960, 1961). However, discussing another oligophagous caterpillar, *Protaparce sexta*, Waldbauer and Fraenkel (1961) comment that odours from most plants cause the larvae to bite on confronting almost any leaf. Biting here appears automatically to follow orientation to olfactory stimuli common to many plants.

Hungry insects often bite with apparently no other stimulus than contact with a biteable substrate. Indiscriminate attacks on such unlikely materials as metals, glass, wax and wood were noted with hungry locusts (Dadd, 1960b) and it is entirely conceivable that phytophagous insects, particularly those which feed at leaf edges, might normally require no more than contact with a suitable surface to initiate feeding. Vision might assist in this; in discussing the attraction of grasshoppers to vertical lines it was noted that attempts at biting were frequently made when following such lines, as also when walking up blades of grass (Williams, 1954).

Whether biting is a discrete activity or a concluding phase of successful orientation it is likely to be non-specific with respect to food plant species in many acridids, for frequently they have been observed to bite unsuitable food-plants before rejecting them (Clark, 1948; Pfadt, 1949; Johnston and Buxton, 1949; Williams, 1954). These observations, incidentally, demonstrate the importance of gustatory stimuli in food discrimination; apart from those instances where repellent odours are encountered during orientation and before biting (Williams, 1954), acceptability is probably determined mainly at this stage. *Schistocerca* hoppers rejected maize seedlings at a particular stage of growth although older plants were acceptable; other extracts of maize of all ages and the foliage of many other plants induced biting when applied to filter paper, but whereas this also occurred with acetone extracts of all foliage excepting young maize, extracts of the latter inhibited biting (Goodhue, personal communication). Although not eaten, rejected seedlings often bore mandibular impressions, suggesting that a repellent was sensed as the first bite released juices or odours from crushed tissue which up to that point had been attractive. Similar studies (Chauvin and Mentzer, 1951) showed that aqueous extracts of two plants, *Melia azedarach* and *Scilla maritima*, which are rejected by many grasshoppers, inhibited starved adult *Schistocerca* from eating moist filter paper or barley seedlings. The active substance of *Scilla* was the bitter glycoside, scillaren, but otherwise little is known of the identity of feeding inhibitants.

More work has been concerned with the identification of phagostimulants. Wheatbran, long used as an attractive basis for grasshopper baits, yielded an ethereal extract which stimulated adult *Schistocerca* to eat filter paper impregnated with it (Chauvin, 1951). The phagostimulant was distinct from volatile components presumably responsible for the attraction of *Schistocerca* to bran from a distance as a steam distillate failed to induce feeding. Chauvin (1951) also found the oils of ground nuts, olives and *Melaleuca viridiflora* (Miaouli oil) to be feeding

stimulants; after saponification, only the fatty-acid fraction of olive oil remained active, but four fatty acids (palmitic, oleic, stearic and undecylic) were devoid of stimulant properties. An ether extract of bran also induced *Schistocerca* hoppers to feed; no response was detected with olive oil, but wheatgerm oil induced feeding (Dadd, 1960c). Recently the phagostimulant action of wheatgerm oil on various grasshoppers was found to be a property of its phospholipids (Thorsteinson and Nayar, personal communication).

Sugars commonly induce insects to feed. This was so of a wide range of concentrations of sucrose and glucose with several North American grasshoppers (Thorsteinson, 1958a). Sucrose, glucose, fructose, maltose and soluble starch proved strongly phagostimulatory to *Schistocerca* larvae; lesser responses were induced by lactose, mannose and sorbose, doubtfully by galactose, and none by xylose. All these sugars with the exception of galactose, xylose and sorbose are of nutritive value to *Schistocerca* (Dadd, 1960d, e).

Other water soluble compounds likely to occur in plant tissues, among them ascorbic acid, thiamin, betaine, monosodium glutamate, various amino acids, oxaloacetic and citric acids, and potassium hydrogen phosphate, stimulated feeding in grasshoppers (Thorsteinson, 1958a, 1960). The effective concentrations of some of these substances were very critical, and effects were sometimes only apparent in certain combinations or with sugar; although none were as effective as sugar it is nevertheless conceivable that in the absence of powerful phagostimulants the integrated effect of such minor stimuli might be important, a view supported by the fact that *Schistocerca* fed well on synthetic diets consisting solely of materials for which no pronounced individual phagostimulant effects could be demonstrated (Dadd, 1960e).

3. *Endogenous factors in feeding*

Suitable stimuli will induce feeding only in hungry insects. As hunger is fostered by starvation it is not unnatural to suppose that it depends upon internal nutritional demands. In the blowfly *Phormia regina*, the only insect in which hunger has been critically studied, this is not obviously so; feeding was uninfluenced by blood sugar, stored glycogen, or gut contents (Evans and Dethier, 1957), and Dethier and Bodenstein (1958) supposed it to be controlled by a neural mechanism localized in the foregut. However, Evans and Brown (1960) found this explanation inadequate and thought a hormonal factor might influence feeding as in the Colorado beetle (De Wilde and Stegwee, 1958). In this case, it is conceivable that hunger may be related to the general metabolic regula-

tion in so far as this is influenced by and influences endocrine activity (Van der Kloot, 1960; Gilbert and Schneiderman, 1961). Appetite, meaning those endogenous factors which govern the amount of feeding, is evidently influenced by internal nutritional demands in another fly, *Calliphora erythrocephala*, for different quantities and types of food were selected during egg maturation, oviposition and after humoral manipulation (Strangeways-Dixon, 1961a, b).

In *Phormia* mobility increases with hunger (Browne and Evans, 1960). This occurs also in starved locusts (Edney, 1937; Ellis, 1951; Chapman, 1954, 1955), and by enhancing the probability of encountering food is obviously advantageous to a hungry insect. A connection between nutritional status and hunger is implicit in a relationship between ingested potassium and locomotory activity in *Locusta*. Blood potassium was lower in starved than in grass-fed locusts and this could increase the responsivity of muscle (Hoyle, 1954); further, the ingestion of potassium reduced marching in *Locusta* hoppers (Ellis, and Hoyle, 1954). However, although *Nomadacris* is likewise more active when hungry, no correlation could be found between the potassium levels of its blood and its food (Chapman, 1958).

Caged adult *Schistocerca* daily consume only half the weight of fresh grass (Davey, 1954; Norris, 1961) estimated to be eaten by those flying in swarms (Weis-Fogh, 1952), presumably because the high metabolic demands of flight entrain increased appetite. *Schistocerca* hoppers eat most at mid-instar, and adult food consumption falls with age; the amounts consumed by isolated and crowded males differ, both before and after maturation (Davey, 1954; Singh, 1957; Norris, 1961). The different physiological states which these appetitive changes reflect doubtless entail varying nutritional demands.

If appetite reflects internal nutritional demands it may be expected that the utilizability and amount of food eaten would be related. The utilization of grass by *Schistocerca* fell from 78% in the first instar to 35% in the fourth instar and later stages (Davey, 1954), but as all stages consumed equal amounts per unit bodyweight this shows only that digestive efficiency changes during development. Utilization also decreases with development in *Melanoplus bilituratus*. The average utilization over the whole growth period of three food plants was identical (32%) and similar quantities of two of them, wheat and grass, were consumed; less oats was eaten, apparently because it was distasteful and in consequence growth was stunted (Smith, 1959). The grass appeared to be deficient in some nutrient of crucial importance during the first instar, and the markedly greater amount of it consumed at this

stage was perhaps an appetitive response to an unsatisfied nutritional demand.

The utilization by *Schistocerca* and *Locusta* larvae of synthetic diets containing different proportions of indigestible cellulose was studied under conditions which excluded the possibility that a requirement specifically for water (Norris, 1959, 1961) might affect food consumption (Dadd, 1960e). Diets consumed in greatest amounts were utilized least, and values calculated for the amounts of utilized food were similar for bran, grass and all synthetic diets. Under the standardized conditions of this experiment appetite was clearly regulated by the nutritional value rather than the quantity of food consumed.

C. THEORIES OF FOOD-PLANT SELECTION

The designation of insects as monophagous, oligophagous or polyphagous arose from the belief that some eat only one species of plant, some a limited and usually related group, and others many taxonomically disparate species. The terms were in origin discriptive of ecological observations, and only latterly has some insight been gained into the physiological mechanism of food selection underlying them. Concurrently more critical ecological study has obscured the numerical distinctions originally implied, as the examples of the feeding habits of grasshoppers (once the prime example of indiscriminate polyphagy) make plain. At the other extreme the supposedly monophagous silkworm may be reared on several plants besides mulberry (Legay, 1958). It seems likely that strict monophagy is a rarity and that most polyphagous insects probably exercise some discrimination.

The species of plants used as food are largely determined by propinquity, for, by reason of geographical distribution or seasonal occurrences, only a limited flora will be available to any insect (Hering, 1950, 1952; Dethier, 1953, 1954a; Painter, 1936). Foodplant limitation of this sort, in that it may give rise to attributions of monophagy and the rest, is a possible source of confusion when these terms are used in relation to the active food selection here in question.

Some of the behavioural mechanisms governing patterns of food selection emerged from the demonstration that the food-plants of particular oligophagous insects contained in common specific substances which provided olfactory or gustatory stimuli without which feeding failed to occur (Dethier, 1937, 1941; Thorsteinson, 1953a). Lacking evidence of alternative mechanisms, these cases were considered typical of mono- and oligophagous insects as a whole, and led to the view that

feeding was governed by special substances, variously termed acceptants, attractants, token factors, or phagostimulants (Dethier, 1947, 1953; Fraenkel, 1953, 1956; Lipke and Fraenkel, 1956). Dethier (1947) suggested that monophagy was best defined in terms of a need for one specific chemical attractant (or several confused by the insect as one), and oligophagy in terms of a need for one of a few such factors; polyphagy is then shown by insects which need no special inducements to feed and therefore accept anything not distasteful or repellent.

This view is no longer wholly tenable for reasons which are discussed at length by Kennedy (1953, 1958) and Thorsteinson (1953b, 1958a, b, 1960). Amongst them are the facts that whereas nutrients are often found to possess phagostimulant properties for both polyphagous insects and those which are strictly oligophagous, no special phagostimulants are known for certain noted oligophagous insects which are, however, influenced by feeding inhibitors. Thorsteinson (1960) cites the Colorado beetle as an example of this situation and proposes an hypothesis of food selection which in some ways is a reversal of that hitherto accepted. Feeding, he suggests, is primarily stimulated by universally distributed plant substances including nutrients and water, but various degrees of oligophagy result from the presence in some plants of factors which inhibit feeding. An important feature of the hypothesis is the prominence given to the probability that most phytophagous insects derive some stimulus to feed from generally distributed substances, including nutrients. This is not to imply that nutrients are recognized as such, but is an acknowledgement that in most experimental situations nutrients have been needed to induce optimal feeding responses. The emphasis on nutrients commends Thorsteinson's scheme as a framework for appreciating the feeding behaviour and food preferences of acridids, for it conveniently embraces the facts that most substances found to stimulate their feeding also have nutritional value. Moreover, it accommodates the many observations which suggest that food-plant preferences may be influenced by nutritional factors.

Many oligophagous insects, including some grasshoppers (Isely, 1944), will not eat unusual plants even under stress of extreme starvation, whereas others, even though markedly fastidious when unstressed, will do so. If oligophagy results from a need for single specific substances (Dethier, 1947), their contribution to overall stimulation is likely to be great and in their absence the reluctance to feed extreme. On the other hand, should oligophagy be the outcome of a variety of inhibitory factors, some of which are postulated to be present in all except the limited number of plant species preferentially eaten (Thorsteinson, 1960), the

degree of inhibition attached to any one of them might be low. It could thus easily fall below threshold during starvation and so allow feeding on unusual plants. The degree of reluctance to feed eccentrically when starved might thus provide a means of distinguishing between these alternative types of oligophagy. On this view, the rigid refusal of graminivorous Acrididae to eat forbs would indicate a stringent requirement for a factor limited to grasses. This surmise apart, it is clearly the case that many grasshoppers do not require a taxonomically specific phagostimulant and are prone to lose their fastidiousness on starvation, a circumstance which has proved of the utmost convenience in some nutritional studies discussed in ensuing sections.

II. Nutrition

A. General Considerations

Nutritional information is obtained from two distinct types of enquiry. On the one hand, food and excretory material may be subjected to chemical analysis and from a comparison of the results certain dietetic needs can be inferred; on the other, different natural and artificial foods may be compared for their ability to maintain growth and development. The second method affords direct evidence of nutritional needs, the characterization of which depends upon the precision with which the composition of the foods is known. Of these methodological approaches to nutritional investigation the analytical is embodied to some extent, if only by implication, in all studies which are more than statements of good or bad foods. It will be apparent on reflection why this must be so, for differences in growth on various natural or artificial foods can afford little nutritional insight without some knowledge of the composition of the foods concerned. Indeed, to be at all practicable the formulation of synthetic diets with the intention of determining requirements for nutrients by their systematic omission presupposes prior information on at least the approximate constitution of the normal food. Fortunately for the entomologist a century of preceeding work on vertebrate nutrition has made available the more fundamental analytical information. It is generally accepted that insects, in common with other invertebrates, require much the same major categories of nutrient as do vertebrate animals. Nothing so far gives one cause to doubt the basic truth of this. The major concern of insect nutrition is therefore to detect the small but important qualitative differences, or to assess the often large quantitative differences in which they differ amongst themselves and from other

animals, and relate these so far as is possible to peculiarities of physiology and ecological situation.

An important area of study in vertebrate nutrition which receives little attention from entomologists concerns the critical assessment of the effects that comparatively marginal changes in the absolute and relative amounts of the major nutrients (protein, fat and carbohydrate) may have on growth. The silkworm is a notable exception on which much information of this type has accumulated (Legay, 1958; Yokoyama, 1963). That an incentive to such studies may sometimes arise with certain pest insects is suggested by work on the survival and fecundity of grasshoppers reared on wheat in which various levels of nitrogen were induced by suitable hydroponic culture (Smith and Northcott, 1951). On wheat made to contain less than 4% N (dry weight) none lived to become adult. In a later paper (Smith, 1960) it was commented that this level of nitrogen is not infrequently met with in some normal field-grown wheats, so that the possibility of finding "resistant" wheat varieties suitable for humans but inadequate as protein sources for grasshoppers might bear consideration. That plant resistance might have a nutritional basis (an "antibiosis" type of resistance (Painter, 1936)) appears feasible from studies on the pea aphid which relate its growth on resistant and non-resistant varieties of pea to differences in their sugar–nitrogen ratios and levels of free amino acids (Maltais and Auclair, 1957; Auclair *et al.*, 1957).

Studies aiming to show which major nutrients are depleted from the normal food by comparing its composition with that of the faeces pre-eminently exemplify the analytical approach. By such means the relative importance of protein and various classes of carbohydrate in the food were investigated in leaf-eating caterpillars (Brown F. M., 1930; Evans, 1939a, b; Crowell, 1941), in the mealworm (Evans and Goodliffe, 1939) and in the grasshopper, *Melanoplus bilituratus* (Brown, A. W. A., 1937a, b). Studies such as these are evidently a more detailed extension of the sort of work on overall utilization discussed in a preceding section. Overall utilization can vary considerably during an insect's developmental history and it is to be expected that this may involve relative changes in the utilization of different nutrients, as had indeed been demonstrated clearly in the European corn borer, *Pyrausta* (Beck, 1956), and tentatively in two locusts, *Schistocerca* and *Locusta* (Dadd, 1960d). An analytical approach is likely to provide the best means of examining this sort of situation without introducing complications due to appetitive changes or nutrient imbalance that may arise from growth studies on diets containing different levels of the nutrients being examined.

Undoubtedly, qualitative or overall quantitative requirements are best ascertained by the use of foods which can be made deficient in particular nutrients at will. Most of the considerable body of nutritional information for insects has been acquired during the past two decades by such methods, particularly as synthetic and more or less precisely defined diets have been made available for an increasing number of species (Trager, 1953; Lipke and Fraenkel, 1956; Levinson, 1955; Friend, 1958; House, 1958, 1961, 1962).

For this type of work experimental foods need not necessarily be synthetic. When extractive procedures can be applied to natural foods to eliminate specific putative nutrients while leaving others essentially unchanged, requirements for those eliminated can be investigated; this method has found extensive application in the study of sterol requirements and their utilization in several insects (Ishii, 1955; Levinson, 1958, 1962) and has been employed in attempting to show a nutritional function for ascorbic acid and carotene in the silkworm (Murthy, 1953, quoted by Friend, 1958). Natural foods can be used without treatment if by suitable culture they can be obtained so as to be deficient in specific substances. This approach has obvious attractions for use in studying insects which cannot be induced to feed normally on synthetic foods, notably those which are phytophagous. Its application to the study of the nitrogen requirements of grasshoppers has already been quoted (Smith and Northcott, 1951) and the method was extended to examine the affect on the same species of different amounts of phosphorus in the plant (Smith, 1960).

With synthetic diets consisting of highly purified components the composition of the food can be made constant and is known with a precision limited only by the impurities in the components used and the extent to which contamination is avoided during the course of experimentation. By the omission of single components from such diets specific requirements can be demonstrated beyond doubt, the magnitude of the requirements assessed by the use of diets containing graded dosages and their interactions with other dietary components followed by concurrent manipulation of these. However, this is not always the simple matter it would at first seem.

Contamination is likely to result from the synthesis of certain nutrients by micro-organisms which invade experimental diets; this frequently necessitates the development of aseptic techniques for preparing diets, the sterilization of eggs of experimental insects, and the maintenance of developing larvae in axenic culture thereafter. The various circumstances in which such techniques are essential, or can be

dispensed with, are discussed fully by House (1961) and Fraenkel (1959). In dealing with comparatively large and active insects such as locusts and grasshoppers axenic rearing was not found practicable. A full discussion of the bearing of this on the interpretation of the results of growth experiments with defined diets is given by Dadd (1960b).

Assumptions about the purity of ingredients used in conducting diets are a more likely source of interpretative error as Gordon (1959) has emphasized. This is more particularly so of conclusions drawn about the inessentiality of vitamins and trace minerals which may easily be introduced as unaccounted contaminants in major components. An instructive illustration of the possible consequences is provided by the discrepant findings relating to the carnitine requirement of *Tenebrio molitor*; their eventual resolution involved the recognition of differing racial requirements for carnitine, the dependence of the expression of a carnitine deficiency on satisfactory mineral nutrition, the contamination of some samples of casein by carnitine and also by amounts of zinc sufficient to obscure the effects of inadequate salt mixtures, and the discovery that a particular commercial salt mixture differed grossly from its supposed composition (Fraenkel, 1958). The difficulties involved in purifying dietary materials sufficiently to rule out all possibility of their contamination by factors, especially trace minerals, which might possibly have nutritional significance is discussed very fully by Hutner *et al.* (1961). They point out that as a practical undertaking such purification is only feasible for occasional, specific purposes. This being so, it will generally be the case that certain nutritional requirements could remain unappreciated, and caution should be exercised before making categorical pronouncements on what is *not* needed.

The possibility that reserves of particular nutrients might initially be present in the experimental insect in amounts sufficient to allow growth to proceed normally for some time is another fertile source of erroneous attributions of dispensability. Locusts allowed to feed on grass for the first two instars could thereafter develop without ascorbic acid or carbohydrate, although both are necessary when reared on synthetic diets from hatching (Dadd, 1960d, b). In the same insects maternally-derived reserves of carotene in the egg were found to mask effects on pigmentation brought about by a subsequent dietary deficiency (Dadd, 1961c). To avoid such carry-over of nutrient reserves, growth studies ideally should be continued through the adult reproductive period and subsequent generations. By so doing Gordon (1959) was able to reveal previously unrecognized requirements in the German cockroach. However, the additional time involved is a deterrent to this degree of

thoroughness, and in practice most students have been content to follow events through the larval growth period only. It is to be remarked that many insects reared satisfactorily to the adult stage on synthetic diets may, in any case, be unable to mature and reproduce. While this remains the situation for a particular species, it must be considered likely that the diet lacked some unspecified nutrient which was either of significance in reproduction specifically or was present in the egg in sufficient amount for a requirement not to become evident until the adult stage.

B. SPECIFIC REQUIREMENTS

1. *Proteins and amino acids*

The work of Smith and Northcott (1951) showing that growth and survival of *Melanoplus* were adversely affected when the nitrogen content of its food-plant was reduced by suitable culture allows an inference of the protein requirement for normal growth if the usual assumption is made that most of the nitrogen was present as protein or related amino compounds. From their data it would appear that little development was possible beyond the second instar with only 21% protein, while growth was retarded and survival poor with 27%; the normal expectation of survival occurred with nitrogen equivalent to 39% protein. For the same species a synthetic diet which contained 20–25% of protein (as casein and yeast protein) allowed up to 90% of first instar larvae to develop to the adult stage (Kreasky, 1962). Satisfactory synthetic diets for the locusts *Schistocerca* and *Locusta* generally contained about 27% protein (Dadd, 1960b) but as little as 20% or as much as 40% could be used without apparent detriment (Dadd, 1960e, 1961a). Proportions of protein of the order of 30–40% have been satisfactory in other orthopteroid insects (Noland and Baumann, 1951; McFarlane *et al.*, 1959).

Casein, because of its ready availability in standardized form, is the protein most frequently used in compounding insect diets. Highly purified "vitamin-free" casein was quite inadequate for the growth of *Locusta* larvae and unsatisfactory for *Schistocerca*. A mixture of casein, egg albumen and peptone allowed both species to develop satisfactorily, but whereas a soluble grade of casein was as good as this mixture for *Schistocerca* it was not so for *Locusta*. Various attempts to offset the inadequacy of purified casein by supplementing it with various amino acids, additional vitamins, plant ash and nucleic acid, certain of which might conceivably have been present in the cruder proteins, were to no avail (Dadd, 1960b, 1961b). *Locusta* and *Schistocerca* also differed in

their ability to grow on diets in which a mixture of twenty amino acids was used to replace all protein. Such diets allowed *Schistocerca* to develop to the adult stage, although at a poor rate of growth, but failed to support *Locusta* beyond the second or third instar (Dadd, 1961b).

No wholly satisfactory explanation of these observations could be given. It is not altogether surprising that casein, an animal protein, should be unsatisfactory for plant-eating insects, but it is difficult to understand why in this case the admixture of further animal protein (albumen and peptone) should be satisfactory. It was at first supposed that an unrecognized growth factor might be in question, or that critical difference might have existed in the amino acid balance of the various proteins used (Dadd, 1960b). Several authors have stressed the sometimes gross effects on growth which quite moderate changes in the amino acid composition of diets may have (House, 1959; Davis, 1959; Vanderzant, 1958; Friend *et al.*, 1957). To support this latter suggestion the possibility has been discussed that differential digestibility of proteins might radically alter the proportionality of available amino acids from that existing in the undigested protein (Dadd, 1961b). By further supposing that the two species possessed digestive enzymes of different characteristics this explanation could be extended to encompass the different growth responses of *Locusta* and *Schistocerca*. However, differential digestibility could not account for the strikingly different responses of the two species to the amino-acid containing diets and an explanation involving the hypothesis of undetermined growth factors assumed to be required to a different extent by the two species was thought to cover the situation more aptly.

Three synthetic diets which allowed satisfactory growth of certain other insects failed to support the development of *Melanoplus* spp. beyond the second instar, although one of them when modified by the addition of yeast and an aqueous extract of lettuce, became a satisfactory diet for growth to the adult stage (Kreasky, 1962). It is unlikely that the small contribution to the protein of the diet made by the yeast can have markedly changed the overall amino-acid balance and this suggests again that the need for it is to be attributed to some growth factor otherwise lacking.

2. *Nucleic acid and nucleotides*

So far no insect has been found with an absolute requirement for nucleic acid or its derivatives, although the growth rate of certain dipterous larvae may be somewhat increased by their inclusion in the diet (Lipke and Fraenkel, 1956; House, 1958). It has been suggested in

these cases that larval growth is so rapid that the ability to synthesize nucleotides is a limiting factor that restricts the most efficient expression of other metabolic systems connected with growth unless an extraneous source of purine is available from the diet (Gordon, 1959).

Yeast nucleic acid seemed to be without effect on the growth of *Schistocerca* and *Locusta* (Dadd, 1960b, 1961b). Nucleic acid and a number of nucleosides and nucleotides were found not to improve the growth of *Melanoplus* on a synthetic diet lacking a necessary lettuce extract and neither did the nucleotides of the lettuce extract itself (Kreasky, 1962). Although the unknown lettuce factor required by *Melanoplus* was clearly not a nucleotide it cannot be said conclusively that such substances were without significance in the basic diet as it contained yeast and, therefore, presumably some yeast nucleic acid. Neither yeast nor nucleic acid was required in a satisfactory diet for another orthopterous insect, the house cricket *Acheta domestica* (McFarlane *et al.*, 1959), and it may reasonably be concluded that this category of substances are of no nutritional significance to orthoptera.

3. *Carbohydrates*

Carbohydrate is important in nutrition primarily as a source of energy and a need for it in the diet depends inversely upon the ability and speed with which other bulk nutrients (fat and protein) can be converted to appropriate intermediates that may enter the various cycles of transformation involved in energy metabolism. Many insects are able to effect such conversions with great efficiency and, therefore, have little need for dietary carbohydrate, although it may be utilized if available. For many dipterous larvae protein is a completely sufficient dietary source of energy (House, 1958), and caterpillars of the Waxmoth (*Galleria mellonella*) may be grown on carbohydrate-free synthetic diets consisting largely of protein and beeswax, a complex lipid mixture (Dadd, unpublished results). In striking contrast many adult flies and moths derive their energy solely from carbohydrate and are completely dependent on a dietary source of it, often their only nutritional requirement. Larvae which infest stored products also characteristically require carbohydrate as their major nutrient, but it is possible, living as they do in dry environments without ingestible water, that high carbohydrate utilization may here be connected in part with a need to produce metabolic water rather than calories alone (Leclercq, 1948a).

It may be anticipated that most growing insects will have some facility for drawing on the energetic potentialities of other nutrients when carbohydrates are in short supply. The indications are that many

of the metabolic pathways that allow interconversion of intermediates deriving from all types of major nutrient are present in various insects (Rockstein, 1957; Gilmour, 1961). Some components of the enzyme systems involved have been demonstrated in locusts (Kilby and Neville, 1957; Hearfield and Kilby, 1958; Clements, 1959), and although this information is fragmentary, it suggests that the necessary pathways are available in orthoptera. Nevertheless, the scale of such conversions must evidently be inadequate for more than auxillary purposes in the locusts *Schistocerca* and *Locusta*, which required considerable amounts of carbohydrate in synthetic diets for satisfactory growth (Dadd, 1960d). With 6% or less of either glucose or sucrose in the diet growth was adversely affected from hatching and no larvae survived to become adults. With 13% of sugar, growth was optimal until the third instar, but it declined thereafter and survival to the adult stage was poor. Optimal growth throughout development occurred with 26% of sugar (or a mixture of sucrose and dextrin) and this was taken to indicate an increased metabolic requirement for carbohydrate during later larval life, perhaps in connection with the building up of reserves of fat, akin to the change in carbohydrate requirement recorded for certain caterpillars (Beck, 1956).

As sugars were shown to have marked phagostimulant properties (Dadd, 1960e) it might be thought that these differences in growth were the outcome of differential feeding. However, evidence was offered to indicate that this was unlikely to be the case. Diets totally lacking carbohydrate at first supported the growth of third instar larvae taken from previous grass culture as well as did diets with sugar; under these conditions the deleterious affect of the lack of sugar became apparent only during the last instar. Besides showing that normal amounts of diet were being eaten without the phagostimulant effect of sugar, this suggested that on adequate food such as grass sufficient metabolic reserves could be accumulated during early development to delay the expression of a subsequent carbohydrate lack.

The suitability of various carbohydrates and other substances which might serve in their stead was examined in both species of locust. The pentose sugars D-xylose, L-arabinose, D-ribose and L-rhamnose were utilized for growth by neither species. Of the hexoses, glucose and fructose were well utilized and D-mannose partially used by both species. *Locusta* utilized neither L-sorbose nor D-galactose, but whereas *Schistocerca* failed to utilize the former, there was some indication that growth was better with galactose in the diet than without it. The dissacharides maltose, cellobiose, trehalose, sucrose, lactose and melibiose,

and the trisaccharides melezitose and raffinose, all supported good growth in both species as did dextrins and soluble starch. Development was completed on diets containing potato starch, but at a somewhat retarded rate of growth. The sugar alcohols sorbitol, mannitol and meso-inositol were all utilized to some extent by both species, but whereas *Schistocerca* completed development (although at a retarded rate of growth) with α-methyl mannoside, this substance inhibited the growth of *Locusta*. That dietary protein and fat could not subserve the functions of carbohydrate to any appreciable extent was shown by the failure of both species to grow satisfactorily on diets containing olive oil, glycerol, palmitic or stearic acids or additional casein in place of utilizable carbohydrate.

Locusta and *Schistocerca* are clearly amongst the most versatile of insects in their ability to utilize a variety of sugars and related compounds. In this they resemble the German roach, although totally lacking its ability to utilize glycerol (Gordon, 1959), and stored products (Leclercq, 1948b; Fraenkel, 1955; Bernard and Lemonde, 1949; Lemonde and Bernard, 1953) rather than other leaf eaters (Beck *et al.*, 1949; Hirano and Ishii, 1957). Apparently they differ somewhat in this respect from *Melanoplus*, for analytical studies showed that whereas this grasshopper very efficiently depleted its food of various sugars, it utilized dextrin, soluble starch and glycogen poorly, and various polysaccharides, including starch, not at all (Brown, 1937a, b). These findings, and similar observations based on the analysis of the food and faeces of leaf eating caterpillars (Crowell, 1941; Evans, 1939a, b) accorded with the hypothesis of Hering (1926) that only the simple sugars amongst carbohydrates are of nutritional importance for leaf eating insects. This could possibly result from differential rates of digestion and absorption, leading to selective abstraction of simple sugars when present with polysaccharides. This effect might be more pronounced when, as in the case of starch, the polysaccharides were enclosed in intact plant cells. However, *Locusta* and *Schistocerca* clearly do not conform to this hypothesis in that dextrin and starches seemed well able to fulfil their carbohydrate needs.

The reasons underlying the differential utilizability of carbohydrates as assessed by their effects on growth are worthy of consideration. Failure to be utilized can be envisaged as being due either to poor absorption from the gut lumen or to the lack of the appropriate enzymes for introducing the absorbed materials into the metabolic mill. This latter possibility may perhaps account for the abilities of the various insects which have been studied to utilize monosaccharides and

related small molecules which probably pass through the gut wall comparatively unhindered (Treherne, 1958b; Horie, 1961). Differences in the utilization of more complex carbohydrates have been correlated with the presence of appropriate enzymes capable of hydrolysing them to simple molecules which may then be absorbed from the gut (Fraenkel, 1940, 1955). That this step may be a complex one is indicated by the demonstration in the silkworm that only amylase occurs in the digestive juice, other glucosidases which split many di- and trisaccarides being located in the gut tissue (Horie, 1959); the fact that many oligosaccharides could be utilized for glycogen and trehalose formation must then indicate that they are able to penetrate into the gut epithelium without prior digestion (Horie, 1961). Having been hydrolysed, either in the digestive fluid or after penetration into the gut tissue, the overall utilizability of the original polysaccharide would then be a function of the subsequent metabolism of its components. Gut extracts of locusts were shown to hydrolyse all the oligo- and polysaccharides which improved growth, although whether this was a function of the digestive juice or of intracellular enzymes was not distinguished (Dadd, 1961d). The wide array of sugars which were available to the two locust species is therefore the outcome of broad internal competence to deal with the common hexoses and sugar alcohols coupled with an extensive complement of mid-gut digestive enzymes.

Within small molecules, including those released by the digestion of more complex entities, one might expect subtle differences in utilizability to emerge if rates of intestinal absorption or internal utilization differ. In *Schistocerca* the very rapid absorption of hexoses from the gut by a process which seems to be essentially one of diffusion, facilitated by the rapid conversion of the hexose to trehalose (Treherne, 1958a), does, in fact, differ considerably in overall rate depending on the hexose concerned (Treherne, 1958b). Diffusion through the isolated gut into saline occurred at approximately similar rates for the three hexoses, as might be expected of a true diffusion process; the difference in overall absorption arose from the differential rates of conversion of the hexoses to trehalose (presumably by enzymes of the fat body which have subsequently been shown to be the site of trehalose synthesis (Candy and Kilby, 1959, 1961)) and which thereby affected the concentration gradient between gut contents and haemolymph. Treherne's finding that mannose was less efficiently absorbed than glucose might partly explain the relatively poor growth of locusts on diets containing mannose (Dadd, 1960d); however, although fructose supported excellent growth it was also comparatively poorly absorbed.

The work of Treherne provides good evidence that small carbohydrate molecules may quickly pass unchanged through the gut wall. One wonders whether this may not also be possible for larger molecules such as the dissacharides. A case in point concerns trehalose, which for locusts was an excellent carbohydrate in terms of growth. The digestion tests with extracts of adult *Locusta* guts quoted by Dadd (1960d) indicate that some hydrolysis of this sugar could occur somewhere within the mid-gut or its contents, but against this Candy and Kilby (1959) state that little trehalase is to be found in this tissue. In the silkworm the mid-gut contained the enzymes necessary to digest oligosaccharides and no utilizable oligosaccharides passed unchanged into the blood; on the other hand, α-methyl mannoside and glucoside passed through the gut unchanged to accumulate in the haemolymph because appropriate enzymes were absent from the gut tissues (Horie, 1961). It may be conjectured that the one notable difference found between the carbohydrates available to *Schistocerca* and *Locusta*, relating to α-methyl mannoside, might have arisen because of a lack of the appropriate hydrolytic enzyme in the gut tissue of *Locusta* and its presence in *Schistocerca*.

An interesting insight into carbohydrate nutrition is given by a study of the depletion of reserve materials in hatchlings of *Schistocerca*, *Locusta* and *Nomadacris* which were starved from birth (Blackith and Howden, 1961). In this case the ovarial reserves provided by the mothers are to be thought of as the "food". It was found that the amount of fat at birth was extremely variable and often undetectable; if present it was subsequently metabolized, but its presence or absence was not obviously related to survival. Carbohydrates were found in only trivial quantities. Glucose occurred in the haemolymph in traces which remained undepleted at death and glycogen was absent. Trehalose was depleted during inanition. The initial amounts present were, however, negligible in relation to the total amount of material metabolized during survival and in the formation of the considerable quantity of chitin shortly after emergence. This comparative lack of trehalose in the blood is most surprising in view of its prominence in the later instars and adults of locusts where it forms an important energy reserve (Howden and Kilby, 1961; Bücher and Klingenberg, 1958). Most of the material utilized in maintenance metabolism and in chitin production was shown to be depleted from water-soluble components of the haemolymph, probably certain amino acids and a reducing substance tentatively identified as ascorbic acid. This latter occurred in quantity in the haemolymph of newly hatched nymphs and was completely depleted during subsequent inanition.

The attribution of a gross maintenance function to ascorbic acid is at first sight surprising in view of its usual recognition as a physiologically important but quantitatively minor component of living tissues, more akin to a vitamin than to a gross nutrient. Indeed it was shown to have a function of the latter sort in the nutrition of locusts (Dadd, 1960a). Ascorbic acid proved ineffective as a complete dietary substitute for utilizable carbohydrate for the growth of both *Locusta* and *Schistocerca*. However, in diets containing suboptimal amounts of sugar the inclusion of gross amounts of ascorbic acid improved the growth and survival of both species (Dadd, 1960d). Dadd conceded that these results indicated that ascorbic acid might function to allay the carbohydrate deficiency in *Schistocerca*, but supposed that in *Locusta* the improvement in growth indicated a sparing action of high sugar on the (small) amount of ascorbic acid required as a vitamin. In view of the findings of Blackith and Howden (1961) it seems possible that the beneficial effects of ascorbic acid were in both cases due to a sparing action on the sugar requirement.

4. *Lipids*

With the exception of small amounts of certain sterols and unsaturated fatty acids no insects have been found with an absolute requirement for fat or other lipid material, although a few may grow faster with some in their diet (House, 1958). It might have been thought that the wax moths would be an exception to this, as about half their normal food is beeswax, of which a large portion is utilized (Niemierko and Wlodawar, 1950). However, although recent evidence suggests that wax or fatty acids in the diet may improve their growth (Beck, 1960; Young, 1961), it has been common experience since the demonstration of Haydak (1936) that the wax moth may be reared for successive generations on diets devoid of wax and containing negligible amounts of fat.

In the previous section it was noted that various fatty substances were totally ineffective as substitutes for the large proportion of carbohydrate required in the diet of two locusts, and it is scarcely to be expected that ingested fat would normally be a significant source of energy for acridids as it constitutes only a small fraction of the leafy material on which they characteristically feed. The stored body fat that is the major fuel reserve for the flight of locusts (Weis-Fogh, 1952), apparently metabolized directly by the wing muscles, must presumably be derived from the food mainly as carbohydrate.

Of the lipid factors required in small amounts for growth, a sterol or related substance is without exception essential for all insects that have been the subject of nutritional study. In all cases cholesterol can fulfil

this need. The ability to utilize other natural sterols or various close derivatives of cholesterol shows distinctive differences which may broadly be related to their normal type of food in those insects which have been studied (Levinson, 1955, 1962; Levinson and Bergmann, 1957). Thus although strictly zoophagous species can utilize 7-dehydrocholesterol as well as cholesterol (both of which are usually present in animal tissues), the natural sterols of plants or micro-organisms are only additionally available to species whose normal foods are of predominately vegetable or microbial origin.

Schistocerca and *Locusta* require cholesterol in amounts of the same order as other insects (Dadd, 1960c). In their ability to use natural sterols other than cholesterol they appear to be as specific, although in relation to different sterols, as the strictly zoophagous species. Both species of locusts were able to utilize cholesterol, its acetate, cholestanol, and β-sitosterol (a widely distributed phytosterol) equally well, but not stigmasterol acetate (another plant sterol), ergosterol (from yeast), 7-dehydrocholesterol, cholestenone, 7-oxocholesteryl acetate, lanosterol and a number of other more distantly related steroids (Dadd, 1960c). The non-availability of cholestenone and 7-oxocholesteryl acetate accords with the general failure of these particular structural modifications of the cholesterol molecule to be used by all insects with which they have been tested. More surprising is the complete failure to utilize stigmasterol acetate, ergosterol and 7-dehydrocholesterol, all of which are to some extent available to various other insects whose food is of plant origin. This suggests that the broad distinction between zoophagous and non-zoophagous species may be susceptible of finer subdivision when more information becomes available for other plant-eating types.

That very small structural differences determine the suitability of sterols for a particular species was commented on in a study of the utilization of an extensive series of steroids by the German cockroach (Noland, 1954a). The evidence from locusts further emphasizes this, in that stigmasterol differs from β-sitosterol and 7-dehydrocholesterol from cholesterol only by the possession of an extra double bond, at positions 22 and 7 respectively; ergosterol embodies both these disqualifying features. Noland (1954b) postulated that these fine distinctions in use might be due to the possession by insects of highly specific esterase systems governing selective absorption from the gut, but this has been questioned as a general mechanism on evidence from houseflies wherein the absorption of sterol precedes its esterification (Robbins *et al.*, 1961).

Much work has been devoted to the elucidation of the mechanisms whereby dietary sterols from non-animal sources may be converted to

the sterols typical of insect tissues (Beck and Kapadia, 1957; Clark and Block, 1959a, b, c; Robbins *et al.*, 1962). The insect tissue sterols have generally been assumed to be cholesterol and 7-dehydrocholesterol, but recent work on houseflies indicates that this may not always be so (Monroe *et al.*, 1961; Agarwal *et al.*, 1961). However, despite the element of uncertainty this introduces into the identity of the body sterols concerned, it has been demonstrated, by successfully rearing a beetle which cannot utilize phytosterols (*Dermestes*) on the dried carcases of a variety of leaf-eating insects, that these are all able to effect the conversion of their various food-plant sterols to something resembling cholesterol (Levinson, 1962). The locust *Schistocerca* was amongst the insects used in this work and, in so far as none of its food-plant sterol could be detected in its body, along with the resultant "cholesterol" it was most efficient in effecting the conversion. It is of interest to note that in no case was 7-dehydrocholesterol detected in these leaf-feeding insects, and one wonders whether this has any significance in relation to the unusual apparent inability of locusts to utilize this substance (Dadd, 1960c).

Besides sterols, the only other lipids known to be essential nutrients for some insects are certain poly-unsaturated fatty acids, notably the dienoic linoleic and thrienoic linolenic acids. As these enter into the composition of various lipid phosphatides, representatives of which broad group are probably necessary constituents of all living tissues, it must be presumed that where no dietary need exists the appropriate fatty acids can be synthesized, as in the mealworm (Fraenkel and Blewett, 1947). A dietary requirement for unsaturated fatty acid has been demonstrated in a few species limited to the lepidoptera and orthoptera, amongst them the locusts *Schistocerca* and *Locusta* (Dadd, 1960b). In accord with this, a study of fat synthesis by *Locusta* fat-body indicated that no appreciable poly-unsaturated fatty acids were formed (Tietz, 1961).

Either linoleic or linolenic acid satisfied this requirement equally well in *Locusta* (Dadd, 1961b). This was also true for *Ephestia* (Fraenkel and Blewett, 1946), *Galleria* (Dadd, unpublished observations) and *Pectinophora* (Vanderzant *et al.*, 1957), although in the latter case minimal requirements for the two acids differed considerably, suggesting different methods of utilization. Linolenic acid failed to replace linoleic acid satisfactorily for the German cockroach (Gordon, 1959), but this was possibly due to its rapid deterioration in the diet.

Arachidonic acid, a tetraenoic unsaturated fatty acid of major importance in vertebrate nutrition, was ambiguous in its affect on

Locusta; it perhaps improved growth somewhat, but failed to alleviate a characteristic symptom of fatty-acid deficiency consisting in the emergence of adults with crumpled wings (Dadd, 1961b). It may be noted that faulty pupal emergence or inability to emerge is the chief outcome of fatty-acid deficiency in all the lepidoptera which have exhibited it. Arachidonic could not replace linoleic acid for the German roach (Gordon, 1959) nor for *Galleria*. In the case of *Ephestia* it apparently slightly accelerated growth but failed to prevent wing malformation (Fraenkel and Blewett, 1947), a situation somewhat resembling that which obtained with *Locusta*.

Vitamine E (α-tocopherol) has often been included in the diets of those insects having a fatty-acid requirement. Its omission retarded the growth of *Ephestia*, but this was attributed to an ability, shared by other unrelated anti-oxidants, to protect the fatty acid in the diet from deterioration (Fraenkel and Blewett, 1946). No affect on the growth or development of locusts could be attributed to α-tocopherol (Dadd, 1960b) and such was also the case for *Pectinophora* (Vanderzant 1957).

The fat-soluble vitamins of vertebrates are generally held to have no place in insect nutrition, for incontrovertible instances of a requirement for any of them by an insect are lacking. Equally, it may bear emphasizing that few of the insects that have been studied have been followed through more than one generation to ensure that a carry-over of micro-nutrient reserves could not possibly be involved in such negative findings. As House (1959, 1961, 1962) comments, it is frequently found that a failure to reproduce follows successful larval growth on synthetic diets, a likely explanation for which is the eventual depletion of reserves of some nutrient absent from the seemingly adequate diet.

In a few studies of sterol utilization calciferol (Vitamin D) has been tested as a substitute for cholesterol, always with negative result. It was quite ineffective in such a test with locusts (Dadd, 1960c). However, before too readily dismissing the vitamin D steroids from the sphere of entomological interest it is well to ponder a recent report of the detection in the sterol fraction of cholesterol-fed houseflies of a component having the characteristic structure of a pro-vitamin D (Kaplanis *et al.*, 1960).

Vitamin K, usually as the synthetic pharmaceuticals menapthone or menadione, has likewise been without effect in those few cases where it was incorporated into insect diets. Menapthone was used in combination with various other conjectural accessory factors in sundry attempts to alleviate the poor growth of locusts reared on diets containing highly purified casein as the sole protein, always fruitlessly (Dadd, 1960b, 1961b).

Vitamin A and a pro-vitamin A substance, β-carotene, were reported to improve the growth and survival of the locust *Schistocerca* (Dadd, 1957), but subsequent work with diets of more generally satisfactory nutritional properties failed to support this claim, although the importance of carotene to normal pigmentation was established (Dadd, 1960b). Later, using *Locusta* nymphs from eggs in which the carotene reserves were probably depleted through rearing the mothers on a carotene deficient diet, some slight effects of dietary carotene on growth were demonstrated as well as a major effect on pigmentation (Dadd, 1961c). These results suggest that carotene may require reinstatement as a factor influencing the growth of locusts; the matter will be touched on further in a later section. Here we may note that a need for vitamin A as such remains unproven. No other insects have been shown to require it nor to benefit from it in any way.

5. *Minerals*

A most neglected aspect of insect nutrition concerns the need for inorganic substances. Reasons for this are not far to seek, for of the pitfalls inherent in growth studies with synthetic diets, those deriving from unavoidable impurities in dietary components or from the accumulation of micronutrient reserves in the experimental insects, are likely to intrude most crucially in attempts to determine the need for minerals, particularly when these are anticipated to exert their influence in trace amounts. From the extensive data available on the ultimate composition of insect tissues it is presumed that the major elements needed by vertebrates are probably required by insects also. The fragmentary information we have in general justifies such an assumption. Hence it is usual to provide for minerals in experimental diets by the inclusion of one or other of the popular mammalian nutritional salt mixtures. However, the fact that a need for calcium, an important vertebrate nutrient, could not be demonstrated in *Drosophila* (Sang, 1956) and could therefore have been required in no more than trace amounts, indicates that such mixtures may be far from ideally balanced for insects. This was shown to be so with respect to potassium for the fly *Pseudosarcophaga affinis* (House and Barlow, 1956) and the mealworm (Fraenkel, 1958).

Nevertheless, most insects studied have seemed to grow well enough with these mixtures as their main source of minerals, from which it may be inferred that on the whole they cannot be unduly sensitive to considerable variations in the amounts and proportions of the elements ingested in their food. This would be quite in accord with the notable

homoeostatic powers that enable many insects to maintain a more or less constant concentration of various ions in the haemolymph against a wide range of concentrations in the gut (and also in the external medium of aquatic types), a matter fully discussed by Buck (1953) and Patton (1953). Amongst Acrididae an example of such regulative ability is afforded by the red locust *Nomadacris*; the potassium concentration of the haemolymph was little affected by starvation or by the various potassium contents of several food plants, although it tended to become slightly higher on dessication of the insect (Chapman, 1958).

Dadd (1961b) studied the growth of *Schistocerca* and *Locusta* on diets containing various levels of two nutritional salt mixtures, one of them standardly used in nutritional work with mammals and the other based on analyses of plant ash. The plant-type mixture was not found to be superior in any respect. Both supported optimal growth when present as 1–2% of the dry diet; without salts the insects could not grow and with amounts of less than 1% of the diet growth was retarded, but their incorporation to the extent of 10% of the diet was without deleterious affect. Salt mixtures in amounts of the order of 2–4% of the dry diet were satisfactory for the cricket, *Acheta* (McFarlane *et al.*, 1959), the grasshopper, *Melanoplus* (Kreasky, 1962), and indeed for most insects that have grown on synthetic diets. The beetle *Tribolium* could, like locusts, tolerate a diet of which 10% was a salt mixture (Huot *et al.*, 1957) but adverse effects have usually attended such excessive amounts with other insects.

These observations suggest that locusts are very insensitive to both the overall amounts of mineral in the diet and the relative proportions of the radicals present. This promises to facilitate the practical problems involved in studying single elements or radicals by allowing one to ignore proportional changes brought about in compounding the various salt mixtures deficient in specific elements needed for such work. With this eventual aim in view a simplified mixture of four salts, comprising the eight radicals Na, Ca, K, Mg, CO_3, Cl, PO_4 and SO_4, was shown to allow normal growth of locust larvae (Dadd, 1961b). By appropriate modifications of this basic mixture it should prove possible to assess the magnitude of requirements for the major inorganic elements as has been achieved with the equally mineral-insensitive beetle, *Tribolium* (Huot *et al.*, 1958).

Circumstantial evidence of the effects of certain elements on blood-sucking insects has been inferred by relating their development on different hosts to the ionic content of the host blood (House, 1958).

A similar approach to the nutrition of phytophagous insects reared on living plants has already been discussed, and as an example the reduced survival and fecundity of the grasshopper *Melanoplus* on food-plants cultured so as to contain abnormally high amounts of phosphorus was quoted (Smith, 1959). It seems a little curious that *Melanoplus* should be so adversely affected by a lesser increase in phosphorus alone when *Schistocerca* and *Locusta* can tolerate a tenfold increase above the minimal level in all minerals of their food. One wonders whether the cultural regime that increased the phosphorus of the food-plants may not, at the same time, have induced other changes harmful to the grasshoppers. It is a drawback of all such inferential work based on the analysis of particular components of a natural food that its uniformity, apart from the particular components analysed, is always uncertain. This is not to denigrate such studies, for failing artificial diets they have a valuable place in yielding nutritional hypotheses, and in any case have their own justification in directly assessing the affects of different types of crop management on pest insects.

The optimal concentration of dietary sodium for the cricket *Acheta domestica* was determined by making use of the fact that optimal growth on a diet of dried grass required the addition of sodium chloride (Luckey and Stone, 1960). Certain irregularities in the response to low levels of added salt were noted and ascribed to "hormology", a term which they define as "the study of stimulation and excitation" and which appears to embrace concepts similar to those of homoeopathic medicine. Their results are of interest here in indicating the possibility that mineral deficiency in a natural plant food might be a limiting factor in growth.

Almost nothing is known of the need for trace elements in insects other than those which it can be assumed are essential because of their occurrence in vital enzymes. Because of the disproportionate practical difficulties involved, it is unlikely that much attention will be paid to them unless they force themselves on the attention by sometimes chancing not to be present in diets as contaminants, as in the case of zinc for *Tenebrio* (Fraenkel, 1958). Where "unknown factors" must be supplied in synthetic diets as crude natural preparations, the possibility of their being trace elements can usually be decided by testing the adequacy of the ashed preparation. By this means the "lettuce-leaf factor" required by *Melanoplus* was shown not to be a trace mineral (Kreasky, 1962), and a similar conclusion was drawn for the factors which were deficient in purified casein for locusts (Dadd, 1961b).

6. *Water soluble vitamins*

Schistocerca and *Locusta* were reared satisfactorily on synthetic diets supplied with ten B vitamins and, in addition, substantial amounts of ascorbic acid, the water-soluble vitamin C of mammalian nutrition (Dadd, 1960b). As a requirement for ascorbic acid was unremarked amongst insects until the recent expansion of interest in phytophagous species, its significance for insect nutrition will be considered separately.

For *Schistocerca*, nine of the B vitamins standardly provided in locust diets were shown to be essential. If thiamin, riboflavin, nicotinic acid, pyridoxine, pantothenate or choline chloride were lacking little growth occurred; the omission of folic acid, biotin or inositol, while not preventing growth altogether, so retarded it that few individuals survived to become adults, and those that did so were undersized and feeble. No adverse effects attended the absence of *p*-aminobenzoic acid with this species. *Locusta* grew rather poorly on the particular basic diet used to determine vitamin requirements, as the casein it contained was an inadequate protein for this species; nevertheless it was clear that the same nine vitamins were needed, as indicated by still poorer growth in their absence. There was some uncertainty about the status of *p*-aminobenzoic acid, as in one experiment growth was poorer in its absence (Dadd, 1961a). The cricket *Acheta* also required the nine vitamins undoubtedly needed by locusts; in this case the need for riboflavin as well as for folic acid and inositol was manifested in terms of retarded growth only. The absence of *p*-aminobenzoic acid also tended to delay development, but to a statistically non-significant extent (Ritchot and McFarlane, 1961).

It has been the general experience that all the vitamins mentioned above, with the exception of *p*-aminobenzoic acid and inositol, while not always essential for development to the adult stage, are necessary for the optimal growth of most insects studied. The detailed information on which this generalization rests and the several exceptions relating to particular vitamins for particular species have already been discussed exhaustively in several comprehensive reviews (Trager, 1953; Lipke and Fraenkel, 1956; Hinton, 1956; House, 1958, 1961, 1962). Some cases in which crucially important vitamins are apparently dispensable have been shown to result from their provision by symbiotic micro-organisms (Richards and Brooks, 1958), and in others there is the suspicion that the vitamins thought to be inessential may have occurred as contaminants of test diets or were carried over to the test animals in sufficient quantities in the egg. It is not the intention here to discuss in detail all the B vitamins required by locusts and related insects, for, on the whole,

they are remarkable only for the fact that it was possible to demonstrate a need for so complete a complement of them without the use of aseptic techniques and sterile insects; this affords a considerable justification for Fraenkel's (1959) contention that non-axenic methods still have their usefulness with insects feeding on comparatively dry diets.

However, certain features of the B vitamin, requirements of these orthopteroid insects call for some comment. Hinton (1956) suggested that insects probably synthesize most of the choline they require, for while its inclusion in the diet has improved the growth of most, sometimes in optimal dosages of comparatively enormous magnitude when compared with other vitamins (Lemonde and Bernard, 1955), its omission has not usually interfered drastically with development except in the case of cockroaches. Writing of the German roach Fraenkel and Friedman (1957) state that it is unique amongst species of insects studied in that it requires relatively high levels of choline and that omission of choline from an otherwise adequate diet has a more adverse effect than the omission of any other vitamin. It now appears that this statement may be expanded to include the locusts *Schistocerca* and *Locusta* and the cricket *Acheta*, for choline was absolutely essential for them all. The optimal amount of choline for *Schistocerca* was of the high order required by roaches (Dadd, 1961a) and an inspection of the two vitamin mixtures used with *Acheta* and the comparative growth rates they supported suggests that this was probably also true of this species (Ritchot and McFarlane, 1961).

Gordon (1959) comments that the classification of choline with the B vitamins, as tradition has it, is not justified by physiological or biochemical considerations, since vitamins are known or presumed to function as components of enzymes, whereas choline is required at the higher levels characteristic of a major structural nutrient such as an amino acid. Choline, amongst its several functions, is known to enter into the structure of some of the lipid glycerides (already mentioned in connection with essential fatty acids) and would be better grouped along with other constituents of these as "lipogens".

Another such lipogenic water-soluble growth factor is inositol. Apart from an early suggestion that it was required in the diet of the beetle *Ptinus* (Fraenkel and Blewett, 1943), inositol has, until recently, been found inessential for insects, although it has been usual to retain it in the formulation of insect vitamin mixtures, presumably as a precautionary measure or because of a presentiment of its possible essentiality. This was its status in cockroach studies until recent work in which growth on inositol-deficient diets was followed to adult maturity with *Periplaneta*

(Forgash, 1958), or into the second generation with *Blattella* (Gordon, 1959), revealed a requirement for it in amounts of the same order as for choline. Inositol was necessary for the cricket *Gryllus*; in its absence nymphal development was protracted and adults undersized (Ritchot and McFarlane, 1961). Locusts deprived of it likewise grew poorly and survival to the adult stage was very low; as with roaches, the optimal dosage of inositol was of the high order found for choline (Dadd, 1961a). In addition to these five orthopteroid insects the indispensibility of inositol to the cotton boll weevil *Anthonomus grandis* was recently demonstrated (Vanderzant, 1959), so it is evidently not a requirement of taxonomic significance. One may guess that a need for it may be more widespread than at present appears, for recognition of its importance has only emerged in terms of slightly delayed growth in adulthood or the subsequent generation. Besides affecting growth, inositol deficiency was found to influence the pigmentation of *Schistocerca*; without it melanization was impaired and the yellow colour due to integumentary carotenoids reduced (Dadd, 1961a). The significance of this will be considered in a later section dealing with the affects of nutrition on pigment metabolism.

Besides the ten substances mentioned above the growth of particular insects has been shown to benefit from the inclusion in the diet of a few other water-soluble vitamin-like factors such as carnitine, thioctic (α-lipoic) acid, glutathione and vitamin B_{12}. Carnitine is needed by all tenebrionid beetles so far examined (Fraenkel and Friedman, 1957) but, with the possible exception of the mosquito *Aedes* (Singh and Brown, 1957), has no dietary significance outside this taxonomic group except as a partial substitute for choline. Thioctic acid was required by the onion maggot *Hylemya antiqua* (Friend and Patton, 1956) and glutathione by *Aedes* (Singh and Brown, 1957), but otherwise by no others. Vitamin B_{12} has been claimed to affect the growth of a number of species, sometimes adversely. Of interest in relation to orthopteroid insects, Gordon (1959) recently showed that without it the German roach grew poorly in the second generation. None of these additional vitamins were included in the formulation of the diets used satisfactorily for *Acheta* or the two locusts, but this is not to say that they were not present as trace contaminants nor that a need for them might not have become apparent if growth trials had been extended beyond one life cycle. In view of Gordon's findings with the roach this might well be the case for vitamin B_{12}. However, neither vitamin B_{12} nor the others were able to offset the inferior growth of locusts reared on diets in which the protein was supplied entirely as highly purified casein (Dadd, 1961b).

It is frequently found that for optimal growth on synthetic diets "unknown factors" have to be supplied by the inclusion of small amounts of yeast, plant tissues, or extracts therefrom. This is particularly so for phytophagous insects. In order to rear the grasshopper *Melanoplus* beyond the second instar on synthetic diets it was essential to incorporate dried lettuce powder or an aqueous extract of this material (Kreasky, 1962). The characterization of the active material was carried to the point where minerals, peptides, amino acids and nucleotides could be excluded, and in its other properties it was shown to be very similar but probably not the same as the "unknown factor" required by the European corn borer (Beck, 1953). The strict requirement for this plant-derived factor would at first sight seem to indicate a major difference between the nutrition of *Melanoplus* and the closely related *Schistocerca* and *Locusta*. However, a requirement for some such factor cannot be excluded in the locusts for two reasons. Good larval growth required the use of crude proteins, notably in the case of *Locusta*; this requirement was standardly satisfied by the inclusion of egg albumen and peptone, but small amounts of dried grass meal or grass juice protein were equally satisfactory (Dadd, unpublished observations). Further, satisfactory maturation and reproduction were never achieved, which indicates that the synthetic diets were deficient in some unrecognized way (Dadd, 1960b, 1961b). The fact that the locusts could be grown to adults whereas *Melanoplus* failed at the third instar might represent no more than a difference in the moment of expression of a common deficiency.

C. IDIOSYNCRACIES OF LOCUST NUTRITION

In discussing the food-plant preferences of grasshoppers and locusts the point was made that many observers have commented on the importance of succulent green vegetation in the habitat. A nutritional requirement by the locust *Schistocerca* for something in green-plant tissues may be inferred from the finding that while bran was avidly eaten it was inadequate for rearing hatchlings beyond the third instar (Davey, 1954; Nickerson, 1956). A mixture of bran, yeast and dried milk with about 30% by weight of dried grass meal was also unsatisfactory unless supplemented by a small amount of fresh grass (Howden and Hunter-Jones, 1958). As the amount of grass supplied in this diet was quantitatively trivial its importance evidently lay in the provision of something in the nature of a vitamin. Amongst the vitamins of importance in human nutrition which are particularly associated with fresh plant-food

are vitamin C (ascorbic acid) and vitamin A as the pro-vitamin, carotene. Carotene has been known for some time to be concerned in the pigmentation of locusts and grasshoppers (Grayson and Tauber, 1943; Chauvin, 1941; Goodwin, 1952a) and, commenting on this, Fraenkel (1953) noted that it might have some significance in the nutrition of phytophagous insects. Both ascorbic acid and carotene were subsequently found to have a role in the nutrition of the Desert Locust *Schistocerca* (Dadd, 1957).

1. *Ascorbic acid*

Schistocerca and *Locusta* failed to complete development when reared on synthetic diets lacking ascorbic acid. In *Locusta* the deficiency became most apparent by failure of fifth instar larvae to complete the final moult to the adult stage successfully; in *Schistocerca*, growth was more evidently retarded from the start and the crucial period of heaviest mortality occurred at the moult from the fourth to the fifth larval instar. The haemolymph of grass-reared *Schistocerca* contains reducing material which may be estimated titimetrically with 2,6-dichlorophenolindophenol and is in all probability ascorbic acid. In larvae reared on synthetic diets lacking ascorbic acid this was absent, whereas high levels of dietary ascorbic acid resulted in haemolymph titres comparable with those found for larvae reared on grass (Dadd, 1960a).

Until recently ascorbic acid was generally considered of no nutritional significance for insects, although it was stated to speed the growth of the mosquito *Culex molestans* (Lichtenstein, 1948) and the larvae of the meal moth, *Ephestia* (Fraenkel and Blewett, 1946). For this latter insect dietary ascorbic acid could be adequately replaced by other reducing substances such as α-tocopherol or ethyl and propyl gallates, and hence its function was considered to be the non-specific protection of essential fatty acids from oxidative deterioration. The inability of other reducing substances to replace ascorbic acid in the diet of locusts provided evidence that its function was not that of anon-specific antioxidant (Dadd, 1960a).

Ascorbic acid is probably of universal occurrence in both plant and animal tissues. It has been identified in various species of insects and, in so far as most insects studied have not needed dietary ascorbic acid, it must be presumed that the ability to synthesize it is widespread in the class (Giroud and Ratsimamanga, 1936; Haydak and Vivino, 1943; Joly, 1940; Day, 1949). Sarma and Bhagvat (1942) showed that the rice moth *Corcyra cephalonica* was able to synthesize it, and this was also the case with the cockroaches *Blattella* (Wollman *et al.*, 1937) and *Peri-*

planeta (Roussel, 1958). However, since Caro and Rovida (1939) found that mulberry leaves contained comparatively high concentrations of ascorbic acid many workers, particularly in Japan, have claimed that it had some function in the nutrition of the silkworm (Legay, 1958). Although the fat-body of silkworm pupae was able to effect the synthesis of ascorbic acid from mannose (Gamo and Seki, 1954), it has now been shown by growth studies using synthetic diets that the silkworm requires ascorbic acid for satisfactory nutrition (Ito, 1961). Besides having a nutritional function in the strict sense, it also had a phagostimulant effect on silkworms such that in its absence the amounts of food consumed were reduced and growth thereby depressed. This may be compared with the findings of Thorsteinson (1958a) with grasshoppers. Recently, three more species of phytophagous insects have been reported to require dietary ascorbic acid, and in one of them, the boll weavil, *Anthonomus grandis*, the amount in the body tissues depends upon the concentration in the diet (Vanderzant *et al.*, 1962).

A requirement for ascorbic acid thus appears to be associated with the habit of phytophagy; certain plant-feeding species have presumably been enabled to dispense with the more general ability to synthesize it through having ample amounts in their food. However, although a dependence on dietary ascorbic acid may prove to be characteristic of leaf-eating insects, it is not to be taken as a typical feature of phytophagous nutrition for the requirement is by no means usual amongst the small number of such species examined. For example, whereas the bollworm *Heliothis zea* requires it (Vanderzant *et al.*, 1962), the pink bollworm *Pectinophora gossypiella* does not (Vanderzant, 1957). In this respect the place of ascorbic acid in insect nutrition may be compared with that of vitamin C for vertebrates, amongst which synthetic abilities have been lost in sporadic cases of essentially vegetarian or frugivorous mammals and birds (Chatterjee *et al.*, 1961).

Little can be said of the physiological function of ascorbic acid in insects, but this is scarcely surprising in view of the essential ignorance of its biochemical mode of action in its major function of antiscorbutic in vertebrates (cf. Burns, 1961). In the rat and other mammals it exerts a sparing affect on the requirement for several vitamins of the B complex, partly indirectly via the intestinal microflora, but this is unlikely to have been the case with locusts, as B vitamins were standardly supplied in amounts many times in excess of their minimal requirements (Dadd, 1961a). The possibility that ascorbic acid might be utilized exceptionally as an energy source in maintenance metabolism (Blackith and Howden, 1961) has already been discussed. This can, however, hardly be of

significance except in the absence of more suitable carbohydrates such as occurs immediately following eclosion from the egg. Ascorbic acid has been claimed to be particularly associated with the golgi dicytosomes of neurones of locusts (Moussa and Banhawy, 1960) which would seem to imply for it some importance in neurophysiology.

Some evidence points to the implication of ascorbic acid in the events associated with moulting. In silkworms the amounts present in each larval stage undergo cyclic fluctuations and are minimal during the moulting period (Gamo, 1941). Its concentration in the haemolymph of grass-fed *Schistocerca* larvae varies characteristically during the course of the fourth and fifth instars. Immediately after ecdysis, when the insect is still soft, the titre in the haemolymph is zero; thereafter it steadily increases and remains at a high level from mid-instar until the following moult, after which it is again found to be zero (Dadd, 1960a). Evidently the biochemical reactions involved in moulting or the subsequent sclerotization and melanization of the cuticle entrain the depletion of ascorbic acid from the haemolymph. Fluctuation in certain other reducing values for the haemolymph of fifth instar *Schistocerca* were shown to have a relationship to moulting when due allowance was made for superimposed variation presumed to depend on the nature of the diet (Howden and Kilby, 1960). The principal reducing material was trehalose and this steadily increased from 24 hours after ecdysis (at which time feeding re-commenced) until immediately before the subsequent adult moult. Glucose, a comparatively minor component, followed a similar course. Non-fermentable reducing material thought to be mainly amino acids (Howden and Kilby, 1961) remained at a fairly constant low level until shortly before the adult moult when its concentration trebled. Much of this non-fermentable fraction was accounted as tyrosine, which accords with the general view that this amino acid accumulates in the haemolymph prior to moulting as precursor material for the polyphenols and quinones subsequently involved in cuticular maturation (Sussman, 1949; Buck, 1953).

The detailed biochemistry of sclerotization and melanization of the cuticle is at present a matter of contention. Dennell (1958) suggests that in the formation of blowfly puparia the tanning polyphenols and quinones arise in the cuticle by a process of non-specific and non-enzymatic hydroxylation of amino acids, but it is commonly believed that tanning quinones form from diphenols which have their ultimate origin in the haemolymph by the action of polyphenolase (tyrosinase) on phenolic amino acids (Mason, 1955; Gilmour, 1961). Whatever may be the case for the tanning substances, it is generally conceded that the

precursors of melanin, where melanization is a separate process from sclerotization as in the locust *Schistocerca* (Malek, 1957; Jones and Sinclair, 1958), originate enzymatically in the blood. Now although both phenolase and its substrates are present in the blood and other tissues before moulting, they do not react together except at the appropriate physiological moment and this has led to suppositions that either the enzyme is under inhibition or that it is present as an inactive proenzyme until the crucial moment (Gilmour, 1961). In studying puparium formation in *Sarcophaga* Dennell (1947) found that both tyrosine and tyrosinase were present together in the blood before pupation; the oxidation–reduction potential of the blood which decreased as the larva matured rose sharply at the onset of pupation, and he supposed that this allowed tyrosinase to function at this point. The possibility that ascorbic acid might be involved in maintaining the redox potential at a low, tyrosinase-inhibiting level was considered by Dennell; he was, however, unable to detect ascorbic acid in the haemolymph and later invoked a dehydrogenase system to account for his results (Dennell, 1949). Nevertheless, that ascorbic acid could have an inhibitory function of the sort under discussion is supported by the observation that its topical application to the newly moulted cuticle of the cricket, *Gryllus bimaculatus*, completely inhibited the formation of the normal black melanic pattern (Fuzeau-Braesch, 1959).

Whether cuticular tanning involves the non-enzymatic formation of *para*-quinones by the hydroxylation of phenylalanine and tyrosine (Dennell, 1958) or, in the more orthodox view (Hackman, 1953), the enzymatic formation of *ortho*-Quinones (which Malek (1960) suggested is the case in *Schistocerca*, a view recently supported by evidence that *N*-acetyldopamine is the tanning agent (Karlson and Sekeris, 1962), in so far as all these reactions are critically dependent upon appropriate oxidation–reduction potentials, it is not unreasonable to suppose that changes in ascorbic acid status, occurring when they are in progress, may have some bearing on the poising of a redox system whereby their timing is regulated.

2. *Carotene and nutrition*

a. *Pigmentation.* The often striking colouration of the locust and its tendency to extreme variation within the species has attracted much comment, experimentation and speculation. The earlier work summarized in Uvarov's (1948) review was prone to a proliferation of names for pigments whose identity was not always satisfactorily particularized, but from it emerged the recognition that the colouration of acridids was due

to several chemically unrelated substances, prominent amongst them being yellow carotenoids derived from their food, a characteristic group of reddish brown pigments and, particularly in insects reared on succulent green food, a major green component.

Pigments of these several types first received comprehensive and chemically adequate description in the Desert and Migratory locusts (Goodwin, 1949, 1950; Goodwin and Srisukh, 1949, 1950, 1951) and their contribution to the external colouration of different stages of both *solitaria* and *gregaria* phases was worked out in considerable detail (Goodwin, 1952a). The following points are pertinent to an understanding of the way in which nutrition may affect colouration.

Young larvae of gregarious *Schistocerca* are predominantly pinkish white with a bold black pattern. The pink tinge arises from uncombined insectorubin, an ommochrome pigment, and the black pattern from cuticular melanin. From the end of the third instar the larval ground colour becomes bright yellow owing to the appearance of carotenoid pigments in the integument. Fledgling adults are pink, again because of free insectorubin, but subsequently they become mottled brown, probably by virtue of various protein-insectorubin complexes. On maturation, males become conspicuously yellow because β-carotene migrates into the hypordermis and cuticle. Solitary *Schistocerca*, both larvae and adults, tend to be either uniformly green or buff-coloured. Melanin is virtually absent and insectorubin nearly so. The green pigment characteristic of the *solitaria* phase is an insectoverdin, in this case a mixture of protein complexes of carotenoids (yellow) and a bile pigment (blue), probably mesobiliverdin.

Gregarious *Locusta* hoppers of all instars are black above and shades of brown or orange beneath; the pigments concerned are principally melanin and insectorubin. Carotene is present but contributes nothing to superficial colouration as it is masked by the omnipresent darker pigments. Fledgling adults have cream and purplish brown patterns which darken with age and are attributed mainly to protein-insectorubin complexes, and, as in *Schistocerca*, maturation is attended by the appearance of integumental carotene in the male. *Solitaria* colouration is essentially similar to that of the corresponding phase in *Schistocerca*; however, insectorubin is commonly present and a very weak, diffuse melanization is usual in the adults.

Thus gregarious pigmentation in both species may be characterized by the predominance of insectorubin and melanin, and the *solitaria* type by reduced amounts of insectorubin, little or no melanin in the larvae,

and a tendency for a green insectoverdin to appear. Similar amounts of carotene are found in both *solitaria* and *gregaria* phases, but in the former it is present as a component of the green insectoverdin. The blue mesobiliverdin can therefore be regarded as the typical *solitaria* pigment. Many intermediate types of colouration grouped as phase *transiens* can occur; in these it is to be supposed that insectorubin and melanin, which are perhaps metabolically related (Goodwin, 1952a; Nickerson, 1956), co-exist in various proportions with insectoverdin.

Chauvin (1941) showed that *Schistocerca* reared on food lacking carotene were without carotenoid pigmentation. This was confirmed in a histological examination of larvae of the same species fed on bran, and both melanin and insectorubin, which are topographically associated, were found to be concurrently much reduced in quantity (Nickerson, 1956). As might be expected, the omission of β-carotene from synthetic diets prevented the development in *Schistocerca* of the normal yellow colour in the fourth and fifth instars due to integumentary carotenoids; the appearance of a pink colour in the earlier larval instars and in fledgling adults was also suppressed (Dadd, 1957, 1960b). Were this pink colour due to astaxanthin its absence would require no particular comment, for astaxanthin, a pink carotenoid noted in locusts by Chauvin (1941), is formed in all larval stages by the oxidation of β-carotene (Goodwin and Srisukh, 1949). However, Goodwin (1952a) considered that the pink colour of young larvae and fledgling adults was due to uncombined insectorubin, an ommochrome having no chemical relationship with the carotenoids. From this it would follow that the role of carotene in *Schistocerca* is likely to be more fundamental than the mere provision of raw material for the various carotenoid pigments.

Distinctive differences in colouration could not be found in relation to the presence or absence or dietary carotene in *Locusta* reared on synthetic diets from normal eggs; all diets produced an atypical appearance in that colouration was not that to be expected of the rearing densities used, but rather that described (Gunn and Hunter-Jones, 1952) for very low densities. However, it was otherwise with larvae whose parents had been reared on a special diet presumed to be deficient in carotene. As hatchlings, such larvae were always pallid, unlike the predominantly heavily melanized progeny of gregarious parents reared on grass. Dietary carotene was found to have a remarkable effect on their subsequent colouration. If provided, the larvae became heavily melanized; if absent, although reared gregariously, they remained a light buff colour or became partially blue (Dadd, 1961c). This is essentially the sort of colouration seen in phase *solitaria* if due allowance is

made for the fact that in the absence of carotene only the blue mesobiliverdin component of insectoverdin could be formed.

These differences in pigmentation were accompanied by differences in activity and growth. Larvae fed on carotene-free diets were more sluggish than those given carotene. They grew more slowly, survived less well, and adults, both male and female, were smaller than those allowed carotene in the food. The shape of the pronotum of the carotene-deprived larvae was somewhat curved in the manner of phase *solitaria*, whereas that of the larvae provided with carotene was flat as in phase *gregaria*. However, all adults, including those derived from larvae whose parents were reared normally on grass, had morphometrics tending to the *solitaria* condition.

A similar experiment with *Schistocerca* larvae derived from parents reared on the carotene-deficient diet gave comparable results. Without carotene growth was retarded, and besides the expected absence of pink and yellow colouration, melanization was delayed and incomplete (Dadd, 1961c). In passing, it may be noted that a similar impairment of melanization was found in *Schistocerca* reared on diets lacking inositol (Dadd, 1961a); the connection between this observation and carotene deficiency will be discussed later.

Some evidence was adduced to show that the peculiarities of pigmentation brought on by stringent deprivation of carotene and displayed externally most notably by *Locusta* were present in incipient form in *Schistocerca* of normal gregarious parentage reared on synthetic diets lacking carotene. These often developed a green tinge in the late instars, and it was found that in the fifth instar the haemolymph was usually of a blue colour similar to that observed in the integument of *solitaria*-like *Locusta*. A minority had colourless blood, as did all *Locusta* larvae of comparable antecedents and the majority of *Schistocerca* larvae of earlier instars. This is in marked contrast to the golden yellow blood of *gregaria* or the green blood of *solitaria* or *transiens* phases. By electrophoretic methods it was shown that green blood contained two chromoproteins, one yellow and one blue, which corresponded respectively to the single yellow chromoprotein of yellow blood and to the single blue chromoprotein of the blue blood (Dadd, 1961c). Evidently the blood of *Schistocerca* fed on carotene-deficient diets came to contain the bile pigment mesobiliverdin in spite of crowded rearing densities which, in the case of larvae fed on grass, resulted in the authentic yellow gregarious blood. A blue pigment presumed to be mesobiliverdin was observed in the haemolymph of both solitary and gregarious *Schistocerca* larvae reared on bran (Nickerson, 1956) and it has also been noted to replace

insectoverdin in *Dixippus* fed on radish or potato (Okay, 1953), all of which foods lack carotene.

Now while the appearance of blue pigment in solitary locusts deprived of carotene is unremarkable since mesobiliverdin is characteristic of the *solitaria* condition, its development in similarly deprived gregariously-reared locusts is a matter for comment. Taken in conjunction with the evidence of a concurrent reduction in insectorubin and melanin, of sluggish activity and slower development, and a degree of pronotal curvature, it is suggestive that deprivation of carotene is conducive to the expression of characteristics of the *solitaria* phase. This conclusion may seem surprising so long as the phases are thought of as determined solely by a simple response to rearing density. Although not necessarily implied, the phase designations "*solitaria*" and "*gregaria*", by their emphatically numerical connotation, tend to foster this way of thinking. However, it is coming to be recognized that the criteria of morphology and colouration whereby the phases may conveniently be distinguished are only the superficial expression of complex and fundamental modifications of physiology and behaviour (Uvarov, 1961; Kennedy, 1961), and this being so it is to be expected that various environmental variables may influence them.

Thus while it remains true that in natural circumstances the factor predominantly concerned in channelling overall metabolism in the direction of a particular phase status will be the degree of stimulation deriving from the close proximity of other suitable insects, on the view that the eventual outcome in terms of orthodox phase criteria is but a reflexion of profound physiological changes, it is to be anticipated that particular criteria will be susceptible of modification or inversion independently of external stimuli if suitable artificial means of access to events in the chain of internal metabolic processes becomes available. It seems most probable that in depriving the locust of carotene just such an artificial interference in metabolism is in question. Before pursuing this hypothesis further some consideration may be given to the evidence that factors of the normal environment may sometimes modify the effects of density in determining phase characteristics.

Much of the impetus for studying the influence of the environment on phase criteria came from the observations and opinions of Faure (1932) to the effect that in the migratory and brown locusts the green *solitaria* form arose only in conditions of high humidity and succulent green vegetation, that other solitaries tended to simulate the colour of their habitat, and that the black pattern of gregarious hoppers was caused by the production of "locustine", a hypothetical substance conceived to be

essentially a waste product resulting from the increased activity and metabolism consequent upon mutual stimulation of hoppers developing in crowds. Some confirmation was found that background might influence the colouration of solitary *Locusta*, particularly in the production of dark types (Hertz and Imms, 1937), and many observers, in the field (Johnston and Buxton, 1949; Stower, 1959) and as a result of laboratory experimentation (Gunn and Hunter-Jones, 1952; Okay, 1953; Nickerson, 1956), provided evidence that fresh green food and high humidity are influential in inducing green pigmentation in solitary locusts and grasshoppers.

The melanic pigmentation characteristic of the gregarious phase has likewise been found more labile than considerations of density alone would allow. Husain and Ahmad (1936) showed that the black colour of gregarious *Schistocerca* was more restricted the higher the rearing temperature, a finding confirmed in both *Schistocerca* and *Locusta* by Goodwin (1950), who noted that with increased temperature the production of both insectorubin and melanin was progressively inhibited. Although Goodwin (1952a) supposed that this effect would have no significance in modifying the expression of the gregarious facies over the ranges of temperature met with in the normal habitat, certain types of *solitaria*-like pigmentation and morphometrics observed in field populations have been related to high temperature (Stower, 1959; Stower *et al.*, 1960). From all this it would appear that, even in normal circumstances, far from being determined solely by the number of contacts with other locusts during development, certain of the criteria used to define phase are likely to be contingent on a variety of other environmental influences.

To return to the effects of carotene on pigmentation it is well to note first that while its stringent exclusion seems to induce a tendency for *solitaria* features to appear, it is emphatically not the case that the induction of these requires the absence of carotene, for authentic *solitaria* and *gregaria* locusts contain similar amounts (Goodwin, 1949). Moreover, the succulent vegetation which has been found to favour the extreme, green form is likely to contain above average amounts of carotene. Okay (1953) goes so far as to suggest that fresh green food is essential for the development of green pigmentation in both *Schistocerca* and *Locusta*, as in solitary grasshoppers (*Acrida* sp.), and, furthermore, will cause locusts reared in groups to become green (presumably only to the extent of *transiens* colouration).

Thus any attempt to account for the appearance of *solitaria* characteristics in terms of carotene economy must allow for the fact that as all

locusts normally contain similar amounts of carotene its function cannot be directly regulatory, but is most probably that of an essential link in a chain of metabolic events set in train after regulation has been otherwise determined.

In speculating on ways in which carotene might act to mediate the expression of phase characteristics, vision and humoral function were discussed as spheres of possible implication (Dadd, 1961c).

Vision has been considered an important factor in contributing to the mutual nervous stimulation thought to entrain gregarization when locust larvae become crowded together (Chauvin, 1941; Kennedy, 1956, 1961; Key, 1950; Uvarov, 1961). It can therefore be envisaged that were vision impaired a tendency towards the *solitaria* condition might appear with rearing densities which ought to ensure the gregarious facies. The photosensitive pigments of visual sensory cells have usually been found to contain carotenoids (Goodwin, 1952b). In vertebrates, molluscs and crustacea vitamin A is involved, but until recently the photosensitive pigment of insect eyes were not known although it was generally believed that vitamin A did not occur in insects (Fisher and Kon, 1959). Its apparent absence from locusts prompted the suggestion that astaxanthin, which is abundantly present in their eyes, might have a photoreceptor function (Goodwin, 1952a).

Considerable evidence now indicates that in several insects a photosensitive, retinene-like substance occurs (Goldsmith, 1958; Bowness and Wolken, 1959; Strange, 1961) and, most pertinent to the present discussion, vitamin A itself has been detected in extracts of the eyes of *Locusta migratoria* (Fisher and Goldie, 1961).

Now if, as Goodwin (1952a, b) states, all animal carotenoids are derived from those of plants, the presence of a carotenoid-containing photoreceptive pigment in the eyes of locusts must depend at some point on the ingestion of dietary carotene. This would be so whether vitamin A or astaxanthin is concerned, and the question of whether *Schistocerca* does or does not contain the former is irrelevant at this point. It is thus distinctly possible that stringent deprivation of carotene might impair the vision of locusts, and to the extent that a considerable proportion of the stimulation involved in crowding might thereby be lost, a tendency to develop *solitaria* characteristics might then ensue, even in crowds. A simple experimental situation which might illumine this suggestion would be to determine the influence on phase characteristics of rearing crowded locusts in total darkness. However, although many have speculated on the role of vision in gregarization this information seems to be lacking.

The case for invisaging a connection between carotene function and humoral events rests on the close similarity between the effects of carotene deprivation and those following certain endocrine manipulations. In *Locusta*, the implantation of additional corpora allata, taken from either gregarious or solitary, adult or larval donors, caused a high proportion of gregarious larvae to develop green colouration (Joly, P., 1951; Joly, L., 1954). The similarity to carotene deprivation extended to an effect of implantation on pronotal curvature and adult morphometrics (Joly, P., 1958). A tendency towards *solitaria* colouration followed similar implantations in *Schistocerca* (Joly, P., 1949, 1951; Nickerson, 1956); this change was less marked, but then the solitaria phase is anyway more difficult to produce in this species by the usual method of isolation (Gunn and Hunter-Jones, 1952). Cross injection of haemolymph was found in *Schistocerca* to work in the opposite direction; gregarious haemolymph changed *solitaria* larvae (and implants of *solitaria* integument) in a gregaroid direction, but the reverse did not occur (Nickerson, 1956).

It may be presumed from these findings that the corpora allata either directly produce a factor which induces a tendency to the *solitaria* condition or function indirectly to inhibit a factor tending to produce gregarious features. The latter alternative would most conveniently allow the results of carotene manipulation to be subsumed if it is supposed that carotene were essential for the existence of the hypothetical factor (which for present purposes may be envisaged equally well as either a substance or a specially poised biochemical equilibrium). For then the absence of carotene would necessarily amount to inhibition of the factor, whereas with carotene present the factor would be open to regulation by corpora allata activity. This would embrace the normal situation in which carotene is equally present in all phase conditions. On this view the opposite effects of haemolymph injection fall into place if it is supposed that once in the haemolymph the factor is beyond the ambit of allatum-regulation; for then injection of gregarious blood containing it may affect the *solitaria* insect, but injection of solitarious blood lacking it could have no effect on the *gregaria* phase.

However, this scheme is unlikely to account for the whole story since there is evidence that the ventral (= prothoracic) gland exerts an influence on pigmentation. In *Schistocerca* this gland is considerably smaller in the gregarious than in the solitaria phase. Its partial removal in isolated green solitaries led to their assumption, after subsequent moults, of the black pattern and yellow or pinkish ground colour characteristics of gregarious or *congregans* hoppers (Ellis and Carlisle,

1961). This provides support for Nickerson's (1956) contention that phase colouration in *Schistocerca* is influenced by two endocrine factors; he supposed one to be concerned primarily in background colour and the other in pattern induction, but it seems more likely that background and melanic pigmentation form an integrated system, for both change together under normal phase reversal, both were changed by ventral gland extirpation, and, in so far as carotene is connected with these events, it influences the production of melanin and insectorubin besides the carotenoids and mesobiliverdin of the background.

The prothoracic gland has been implicated in pigmentation regulation in the larva of the moth *Cerula vinula*, where the production of a red ommochrome shortly before pupation requires the presence of α-ecdyson (Bückmann, 1959); premature reddening could be induced in the normally green young caterpillars by decapitation, and it was suggested that in the early instars the *corpora allata* maintained a hormone balance such that the competence of the tissues to produce ommochrome under the influence of the prothoracic hormone (ecdyson) was affected. In the ventral gland operations of Ellis and Carlisle (1961) a change in the *balance* of prothoracic and allatum hormones would necessarily accompany reduction of prothoracic hormone, and it seems most plausible on our present regretably fragmentary evidence to suppose that the regulation of pigmentation, as of other characteristics used as phase criteria, is a matter of humoral balance in which solitariness shows some indication of a preponderant influence of *corpora allata* hormone and can thus be thought of as a somewhat juvenile state (Kennedy, 1956, 1961; Carlisle and Ellis, 1959; Joly, 1958).

b. Growth and reproduction. In discussing lipid requirements reference was made to evidence, admittedly conflicting, that carotene improved the growth of locusts. To help resolve the conflict an effort was made to ensure depletion of the considerable amounts of carotene normally carried over to the egg; the subsequent growth of carotene-depleted larvae was found to be inferior if carotene was absent from their diet (Dadd, 1961c). Now, in as much as stringent carotene depletion has been suggested to bring about a pseudo-*solitaria* condition, slower growth might be yet one more aspect of it, for true solitaries grow more slowly than crowded locusts. However, from consideration of the facts that both males and females were smaller without carotene whereas normal *solitaria* females are larger than female *gregaria*, it was supposed that a general impairment of growth was in question.

That carotene might play some important role in locust metabolism is suggested by the considerable amounts of it in the egg and its meta-

bolism there and throughout later life to astaxanthin (Goodwin, 1949). Goodwin (1952a) speculated that this conversion might ensure that from birth the larvae had photosensitive pigment available for immediate function, but it now seems more likely that photoreception would depend upon retinene derived from vitamin A, at least in *Locusta* (Fisher and Goldie, 1961).

Reproduction is another context in which carotene may be crucial. *Schistocerca* grew to adulthood on a crude artificial diet at a normal rate, but without a small supplement of fresh grass only non-viable eggs were produced (Howden and Hunter-Jones, 1958). As colour abnormalities similar to those due to carotene deficiency occurred on this diet and were rectified by the very small amounts of grass which also allowed fertility, infertility may possibly have resulted from the absence of carotene.

Carotene has some connection with the sexual maturation of gregarious male locusts. At this time they become yellow, due to the migration of β-carotene into the hypodermis and cuticle, and this is accompanied by the production in hypodermal vacuoles of a pheromone liberated over the surface of the cuticle and involved in the sexual stimulation and acceleration of maturation in neighbouring adults. Maturation, translocation of carotene, and the production of pheromone are all controlled by the corpora allata, removal of which prevents or reverses all these functions (Loher, 1960). These concurrent happenings suggested the possibility of some relationship between carotene and the pheromone (Dadd, 1960b) although the two are certainly not identical (Loher, 1960). On diets lacking carotene there is, of course, no possibility that the yellow maturation colour could develop; it generally failed to develop even when the diet contained carotene (Dadd, 1961c). Adults of *Schistocerca* reared and kept for lengthy periods on synthetic diets were only occasionally observed to copulate, whereas those of *Locusta* usually copulated readily shortly after fledging; however, in neither species was oviposition frequent, normal, or the eggs that were occasionally produced viable (Dadd, 1960b, 1961c). As an argument for a role of carotene in reproduction this is somewhat weakened by the fact that reproduction was never obtained whether synthetic diets contained carotene or not. However, there was evidence that even with high dietary concentrations very little carotene was absorbed.

To affect colouration in the ways that have been discussed some absorption of carotene from synthetic diets must have occurred. Nevertheless, in these cases not only was the colour due to carotenoid pigmentation of a lower intensity than is usual in grass-reared insects,

but the fat body, usually intensely yellow or orange, was always white, and the blood, normally bright yellow, was colourless, pale green or blue. Moreover such eggs as were occasionally deposited were pallid and quite lacking the normal yellow tinge. Evidently very little of the ample carotene provided in synthetic diets found its way into the tissues.

This might have been due either to failure of the gut to absorb it properly from the diet or to faulty internal mobilization. In connection with the latter possibility it is relevant that in *Schistocerca* larvae deprived of dietary inositol, melanization was suppressed and the degree of integumentary yellowing reduced (Dadd, 1961a), both of which pigmentary anomalies occur when carotene is absent. What is known of the physiological role of inositol goes to suggest that as a component of phospholipids it is concerned in the mobilization and transport of fatty material (Gilmour, 1961) and its absence might conceivably result in failure to distribute carotene from the gut. The amount of inositol standardly supplied in synthetic diets, although quite adequate for good larval growth, was scarcely up to the minimal level required for optimal colouration (Dadd, 1961a). This may perhaps partly account for the comparative lack of tissue carotene in locusts reared on synthetic diets even when these contained it in concentrations as great as those to be met with in plant tissues. In passing, it is of interest to note that as with other unusual dietary requirements of locusts (unsaturated fatty acids, ascorbic acid and carotene) inositol occurs in great abundance in leafy tissues (Fraenkel, 1953).

When β-carotene seemed to be implicated in the growth of *Schistocerca* it was natural to consider the possibility that it might be a precursor of vitamin A, as in vertebrates. However, the initial indications from growth experiments that this might be the case were not subsequently sustained, and this would accord with the accepted view that vitamin A has no significance in insect nutrition. Nevertheless, reports have appeared suggesting that vitamin A has some function in the silkworm (see the reviews of Lipke and Fraenkel, 1956; Friend, 1958 and Legay, 1958), and there now seems little doubt of its participation in vision. This poses an interesting situation; for if insects, in common with other animals, are unable to synthesize carotenoids *de novo*, as the weight of evidence indicates (Goodwin, 1952b), vitamin A must ultimately derive from their food. Should this be the case it may be conjectured that many of the insects reared on synthetic diets in the course of nutritional studies may have been visually defective. Experiments on the visual sensitivity of insects so reared might be a fruitful if

oblique alternative approach to assessing whether they have a nutritional need for vitamin A.

Should carotene influence growth, it does not, of course, follow that this would necessarily be connected with the provision of vitamin A. Some other derivative might be concerned in growth as distinct from vision. In vertebrates polyisoprenoid structures enter into various molecules of potent biological activity. Besides carotenoids these include the steroids, tocopherols (vitamin E), phylloquinones (vitamin K) and the recently discovered ubiquinones (coenzyme Q). Of these, only sterols are certainly known to be vital for insects, but it is conceivable that substances of these affinities could play a more widespread role than is at present apparent.

Monoterpenoid compounds occur in the defensive secretions of ants and blister beetles (Roth and Eisner, 1962) and a ubiquinone has been detected in insect tissues (Heller, *et al.*, 1960), although whether this has any functional significance is not known. Of more interest from a functional point of view is the recent evidence that farnesol, a sesquiterpene alcohol, can simulate the effects of the juvenile hormone when introduced into certain insects (Schmialek, 1961; Wigglesworth, 1962).

It would not be altogether surprising if substances of terpenoid affinities were involved in insect metabolism. There are indications that this is so of sterols; consideration of the facts that growth is sometimes slightly better with "non-utilizable" sterols than with no steroid at all, and that certain "non-utilizable" sterols have a sparing effect on the requirements for utilizable sterols has been taken to indicate that in addition to being essential structural components of tissues they may provide intermediates concerned in the metabolism of growth and reproduction (Clark and Bloch, 1959b; Monroe *et al.*, 1961; Levinson, 1962; Robbins and Shortino, 1962). Levinson (1962) considers there are similarities between the effects of sterol deficiency and of juvenile hormone deprivation, and to buttress his speculations has pointed out that compounds related to farnesol are currently thought to occupy a position in the sequence of intermediates involved in the biogenesis of sterol. As neither a sterol nor any of these recognized intermediates can be synthesized by insects, the implication here is that should a polyisoprenoid substance be concerned in juvenile hormone function, it may perhaps be derived by the degradation of ingested sterol. This tentative proposition has the attraction of ascribing to cholesterol a fundamental place in insect metabolism commensurate with its probable importance in the nutrition of all insects.

As yet it is premature to assume that juvenile hormone is related chemically to farnesol, even though the similarities between the effects of both substances make this a distinct possibility. However, if it is so related, the metabolism of isoprenoid substances would assume major importance in insect physiology. In this eventuality and should insects prove generally unable to synthesize substances of this sort and so need suitable precursors as nutrients, the terpenoid affinities of carotenoids suggest that they be borne in mind as an alternative source should sterol degradation prove inadequate.

III. Summary Remarks

Throughout this review the intention has been to relate feeding behaviour and nutrition to those other aspects of insect functioning that are sufficiently well surveyed and understood to provide the possibility of integration. A certain amount of this speculation rests on slender justification and doubtless much of it will soon become inconsequential. Even so, notions which may have to be discarded serve some purpose if their delineation throws into sharper focus alternative possibilities which may eventually be seen to approximate more adequately to the true state of affairs. It remains to comment on certain features which, taken together, seem to distinguish the feeding habits and nutrition of grasshoppers and locusts *vis à vis* other insects.

As we have seen, the food preferences of some acridids are strictly limited and a degree of discrimination is usually to be discerned even in those of polyphagous propensities. Nevertheless, many of these latter require little pressure to feed outside their range of native preference, and this may lead to their becoming pests. The indications are that for species of such catholic tastes nutritional factors enter into their choice of food; it is certainly the case that most substances found in laboratory experiments to have a notable effect in stimulating them to feed are of probable nutritional value and of widespread distribution in plants of all kinds. In line with this, plague grasshoppers and locusts tend to depredate a variety of crops, in contrast to perhaps the majority of pest species which are characteristically more or less oligophagous and to that extent a threat only to certain cultivated plants within the taxonomic limits of their oligophagy.

Turning to the nutrition (in the strict chemical sense) of certain locusts, their needs, while qualitatively basically similar to those of the generality of insects, are noteworthy for a few unusual features. These relate to the requirement for ascorbic acid, inositol and unsaturated

fatty acids, and to the critical importance of large amounts of choline in larval growth. A need for the lipogenic factors, choline and inositol, and probably also unsaturated fatty acids, seems to characterize all those orthopteroid insects that have been studied in detail. The requirement for dietary ascorbic acid, on the other hand, appears to be a distinguishing feature of some (but by no means all) plant-eating insects from various taxonomic groups.

The need for dietary carotene to ensure normal pigment metabolism in locusts may be a peculiarity of those particular species in which pigmentation is bound up with the special physiological phenomena of phase. It can be assumed that unless present in the diet the colouration of many other grasshoppers and leaf-feeding insects will also be abnormal. Whether carotene or its derivatives may have a more basic place than this in insect nutrition remains a moot question.

References

Agarwal, H. C., Casida, J. E. and Beck, S. D. (1961). *J. Insect Physiol.* **7**, 32–45. An unusual sterol from houseflies.

Auclair, J. L., Maltais, J. B. and Cartier, J. J. (1957). *Canada. Ent.* **89**, 457–464. Factors in resistance of peas to the pea aphid, *Acyrthosiphon pisum* Harr. (Homoptera: Aphididae). II. Amino acids.

Barton Browne, L. and Evans, D. R. (1961). *J. Insect. Physiol.* **4**, 27–37. Locomotor activity of the blowfly as a function of feeding and starvation.

Barnes, O. L. (1955). *J. econ. Ent.* **48**, 119–124. Effects of food plants on the lesser migratory grasshopper.

Barnes, O. L. and McLellan, C. W. (1960). *USDA Agr. Res. Service Special Report Z*-120. Progress in tests of repellents against grasshoppers, 1960.

Beck, S. D. (1953). *J. gen. Physiol.* **36**, 317–325. Nutrition of the European corn borer *Pyrausta nubilalis* (Hbn.). III. An unidentified dietary factor required for growth.

Beck, S. D. (1956). *Ann. ent. Soc. Amer.* **49**, 582–588. The European corn borer, *Pyrausta nubilalis* (Hubn.), and its principal host plant. II. The influence of nutitional factors on larval establishment and development on the corn plant.

Beck, S. D. (1960). *Wisconsin Acad. Sci., Arts and Letters* **49**, 137–148. Growth and development of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Galleriidae).

Beck, S. D. and Kapadia, G. G. (1957). *Science* **126**, 258. Insect nutrition and metabolism of sterols.

Beck, S. D., Lilly, J. H. and Stauffer, J. F. (1949). *Ann. ent. Soc. Amer.* **42**, 483–496. Nutrition of the European corn borer, *Pyrausta nubilalis* (Hbn.). I. Development of a satisfactory purified diet for larval growth.

Bernard, R. and Lemonde, A. (1949). *Rev. Canad. Biol.* **8**, 498–503. Aspects qualitatifs des besoins en glucides de *Tribolium confusum* Duval.

Blackith, R. E. and Howden, G. F. (1961). *Comp. Biochem. Physiol.* **3,** 108–124. The foods reserves of hatchling locusts.

Bowness, J. M. and Wolken, J. J. (1959). *J. Gen. Physiol.* **42,** 779–792. A light-sensitive pigment from the housefly.

Brown, A. W. A. (1937a). *J. exp. Biol.* **14,** 87–94. Studies on the excreta of a grasshopper (*Melanoplus bivittatus* Say).

Brown, A. W. A. (1937b). *Bull. ent. Res.* **28,** 333–336. A note on the utilization of polysaccharide by a grasshopper.

Brown, E. S. (1947). *Bull. Soc. Fouad Ent.* **31,** 287–306. Egg laying sites of the desert locust in Tripolitania.

Brown, F. M. (1930). *Ann. N. Y. Acad. Sci.* **32,** 221–230. The utilization of hexose carbohydrates by lepidopterous larvae.

Browne, L. B. and Evans, D. R. (1960). *J. Insect Physiol.* **4,** 27–37. Locomotor activity of the blowfly as a function of feeding and starvation.

Brues, C. T. (1946). "Insect dietary; an account of the food habits of insects", 466 pp. Harvard University Press: Cambridge, Mass.

Brunson, A. M. and Painter, R. H. (1938). *J. Amer. Soc. Agron.* **30,** 334–346. Differential feeding of grasshoppers on corn and sorghums.

Bücher, T. and Klingenberg, M. (1958). *Z. angew. Chem.* **70,** 552–570. Wege des Wasserstoffs in der lebendingen Organisation.

Buck, J. B. (1953). Physical properties and chemical composition of insect blood. *In* "Insect Physiology" (*ed. Roeder, K. D.*) pp. 147–217. Chapman & Hall: London.

Bückmann, D. (1959). *J. Insect Physiol.* **3,** 159–190. Die Auslösung der Umfärbung durch das Häutungshormon bei *Cerula vinula* L. (Lepidoptera: Notodontidae).

Burns, S. S. (ed) (1961). *Ann. N. Y. Acad. Sci.* **92,** 1–332. Vitamin C.

Candy, D. J. and Kilby, B. A. (1959). *Nature, Lond.* **183,** 1594–1595. Site and mode of trehalose biosynthesis in the locust.

Candy, D. J. and Kilby, B. A. (1961). *Biochem. J.* **78,** 531–536. The biosynthesis of trehalose in the locust fat body.

Carlisle, D. B. and Ellis, P. E. (1959). *C. R. Acad. Sci. Paris,* **249,** 1059–1060. La persistence des glandes ventrales cephaliques chez les cirquets solitaires.

Caro, L. de and Rovida, E. (1939). *Quad. Nutr.* **6,** 91–96. Vitamin C in mulberry leaves and silkworms.

Chapman, R. F. (1954). *Brit. J. Anim. Behav.* **2,** 146–152. Responses of *Locusta migratoria migratorioides* (R. & F.) to light in the laboratory.

Chapman, R. F. (1955). *Anti-Locust Bull.* **19,** A laboratory study of roosting behaviour in hoppers of the african migratory locust (*Locusta migratoria migratorioides* R. & F.).

Chapman, R. F. (1957). *Brit. J. Anim. Behav.* **5,** 60–75. Observations on the feeding of adults of the red locust (*Nomadacris septemfasciata* (Serville)).

Chapman, R. F. (1958). *Animal Behaviour* **6,** 60–67. A field study of the pottassium concentration in the blood of the red locust (*Nomadacris septemfasciata* Serv.), in relation to its activity.

Chapman, R. F. (1959). *Anti-Locust Bull.* **33.** Field observations on the behaviour of hoppers of the red locust (*Nomadacris septemfasciata* Serville).

Chatterjee, I. B., Kar, N. C., Ghosh, N. C. and Guha, B.C. (1961). *Ann. N.Y. Acad. Sci.* **92,** 35–56. Aspects of ascorbic acid biosynthesis in animals.

Chauvin, R. (1941). *Ann. Soc. ent. Fr.* **110,** 133–272. Contribution a l'etude physiologique du criquet pelerin et du determinisme des phenomenes gregaires.

Chauvin, R. (1951). *Bull. Off. nat. Anti-acrid., Paris* **1,** 15–18. Sur les facteurs responsables de l'attraction que manifestent les acridiens pour le son.

Chauvin, R. and Mentzer, C. (1951). *Bull. Off. nat. Anti-acrid., Paris* **1,** 5–14. Contribution a lé'tude des substance naturalles et de synthese repulsives pour les acridiens.

Clark, A. J. and Bloch, K. (1959a). *J. Biol. Chem.* **234,** 2578–2582. The absence of sterol synthesis in insects.

Clark, A. J. and Bloch, K. (1959b). *J. biol. Chem.* **234.** 2583–2588. Function of sterols in *Dermestes vulpinus.*

Clark, A. J. and Bloch, K. (1959c). *J. biol. Chem.* **234,** 2589–2594. Conversion of ergosterol to 22-dehydrocholesterol in *Blattella germanica.*

Clark, E. J. (1948). *Trans. R. ent. Soc. Lond.* **99,** 173–222. Studies in the ecology of British grasshoppers.

Clark, L. R. (1947a). *Commonwealth of Australia Council for Sci. & Ind. Res. Bull.* no. **226.** An ecological study of the Australian plague locust (*Chortoicetes terminifera* Walk). in the Bogan-Macquarie outbreak area, N.S.W.

Clark, L. R. (1947b). *Comm. of Austr. Council for Sci. & Ind. Res. Bull* no. **228.** Ecological observations on the small plague locust grasshopper *Austroicetes cruciata* (Sauss.), in the Trangie district, Central Western N.S.W.

Clements, A. N. (1959). *J. exp. Biol.* **36,** 665–675. Studies on the metabolism of locust fat body.

Crowell, H. H. (1941). *Ann. ent. Soc. Amer.* **34,** 503–512. The utilization of certain nitrogenous and carbohydrate substances by the southern army worm, *Prodenia eridania* Cram.

Dadd, R. H. (1957). *Nature, Lond.* **179,** 427–428. Ascorbic acid and carotene in the nutrition of the desert locust, *Schistocerca gregaria* Forsk.

Dadd, R. H. (1960a). *Proc. roy. Soc.* (B) **153,** 128–143. Some effects of dietary ascorbic acid on locusts.

Dadd, R. H. (1960b). *J. Insect Physiol.* **4,** 319–347. The nutritional requirements of locusts. I. Development of synthetic diets and lipid requirements.

Dadd, R. H. (1960c). *J. Insect Physiol.* **5,** 161–168. The nutritional requirements of locusts. II. Utilization of sterols.

Dadd, R. H. (1960d). *J. Insect Physiol.* **5,** 301–316. The nutritional requirements of locusts. III. Carbohydrate requirements and utilization.

Dadd, R. H. (1960e). *Ent. exp. & appl.* **3,** 283–304. Observations on the palatability and utilization of food by locusts, with particular reference to the interpretation of performances in growth trials using synthetic diets.

Dadd, R. H. (1961a). *J. Insect Physiol.* **6,** 1–12. The nutritional requirements of locusts. IV. Requirements for vitamins of the B complex.

Dadd, R. H. (1961b). *J. Ins. Physiol.* **6,** 126–145. The nutritional requirements of locusts. V. Observations on essential fatty acids, chlorophyll, nutritional salt mixtures, and the protein or amino acid components of synthetic diets.

Dadd, R. H. (1961c). *Bull. ent. Res.* **52,** 63–81. Observations on the effects of carotene on the growth and pigmentation of locusts.

Davey, J. T. and Johnston, H. B. (1956). *Anti-Locust Bull.* **22.** The African migratory locust (*Locusta migratoria migratorioides* R. & F.) in Nigeria.

Davey, P. M. (1954). *Bull. ent. Res.* **45,** 539–551. Quantities of food eaten by the desert locust, *Schistocerca gregaria* Forsk., in relation to growth.

Davis, G. R. F. (1959). *Ann. ent. Soc. Amer.* **52,** 164–167. Alanine and proline in the diet of the larvae of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae).

Day, M. F. (1949). *Austr. J. Sci. Res.* (B) **2,** 19–30. The distribution of ascorbic acid in the tissues of insects.

Dennell, R. (1947). *Proc. roy. Soc.* (B) **134,** 79–110. A study of insect cuticle: the formation of the puparium of *Sarcophaga falculata* Pand. (Diptera).

Dennell, R. (1949). *Proc. roy. Soc.* (B) **136,** 94–109. Weisman's ring and the control of tyrosinase activity in the larvae of *Calliphora erythrocephala.*

Dennell, R. (1958). *Biol. Rev.* **33,** 178–196. The hardening of insect cuticles.

Dethier, V. G. (1937). *Biol. Bull. Wood's Hole,* **72,** 7–23. Gustation and olfaction in lepidopterous larvae.

Dethier, V. G. (1941). *Amer. Nat.* **75,** 61–73. Chemical factors determining choice of food plants by *Papillio* larvae.

Dethier, V. G. (1947). "Chemical Insect Attractants and Repellents", 289 pp. The Blakiston Co., Philadelphia, Pa.

Dethier, V. G. (1953). *Trans. IXth Int. Congr. Ent., Amsterdam,* **2,** 81–88. Host plant perception in phytophagous insects.

Dethier, V. G. (1954) *Evolution* **8,** 33–54. Evolution of feeding preferences in phytophagous insects.

Dethier, V. G., Barton Browne, L. and Smith, C. N. (1960). *J. Econ. Ent.* **53,** 134–136 The designation of chemicals in terms of the responses they elicit from insects.

Dethier, V. G. and Bodenstein, D. (1958). *Z. Tierpsychol.* **15,** 129–140. Hunger in the blowfly.

Dethier, V. G. and Chadwick, L. E. (1948). *Physiol. Rev.* **28,** 220–254. Chemoreception in insects.

Edney, E. B. (1937). *Bull. ent. Res.* **28,** 243–278. A study of spontaneous locomotor activity in *Locusta migratoria migratorioides* (R. & F.) by the actograph method.

Ellis, P. E. (1951). *Anti Locust Bull.* **7.** The marching behaviour of hoppers of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) in the laboratory.

Ellis, P. E. and Ashall, C. (1957). *Anti-Locust Bull.* **25.** Field studies on diurnal behaviour, movement and aggregation in the desert locust (*Schistocerca gregaria* Forskal).

Ellis, P. E. and Carlisle, D. B. (1961). *Nature, Lond.* **190,** 368–369. The prothoracic gland and colour change in locusts.

Ellis, P. E. and Hoyle, G. (1954). *J. exp. Biol.* **31,** 271–279. A physiological interpretation of the marching of the hoppers of African migratory locust. (*Locusta migratoria migratorioides* R. & F.).

Evans, A. C. (1939a). *Proc. R. ent. Soc. Lond.* (A) **14,** 25–30. The utilization of food by the larvae of the buff-tip *Phalera bucephala.*

Evans, A. C. (1939b). *Trans. R. ent. Soc. Lond.* **89,** 13–22. The utilization of food by certain lepidopterous larvae.

Evans, A. C. and Goodliffe, E. R. (1939). *Proc. R. ent. Soc. Lond.* (A) **14,** 57–62. The utilization of food by the larva of the mealworm *Tenebrio molitor* L. (Coleoptera).

Evans, D. R. and Barton Browne, L. (1960). *Amer. Midl. Nat.* **64,** 282–300. The Physiology of hunger in the blowfly.

Evans, D. R. and Dethier, V. G. (1957). *J. Insect Physiol.* **1**, 3–17. The regulation of taste threshold for sugars in the blowfly.

Faure, J. C. (1923). *Transvaal Univ. Coll. Bull.* **4.** The life history of the brown locust.

Faure, J. C. (1932). *Bull. ent. Res.* **23**, 293–405. The phases of locusts in South Africa.

Fisher, L. R. and Goldie, E. H. (1961). *Proc. 3rd Int. Congr. on Photobiology, Copenhagen* 1960, 153–154. Pigments of compound eyes.

Fisher, L. R. and Kon, S. K. (1959). *Biol. Rev.* **34**, 1–36. Vitamin A in the invertebrates.

Forgash, A. J. (1958). *Ann. ent. Soc. Amer.* **51**. 406–409. The effect of inositol on growth, survival and maturation in *Periplaneta americana* L.

Fraenkel, G. (1940). *J. exp. Biol.* **17**, 18–29. Utilization and digestion of carbohydrates by the adult blowfly.

Fraenkel, G. (1953). *Trans. 9th Int. Congr. Ent. Amsterdam* 1951, **2**, 90–100. The nutritional value of green plants for insects.

Fraenkel, G. (1955). *J. Cell. Comp. Physiol.* **45**, 393–408. Inhibitory effects of sugars on the growth of the mealworm, *Tenebrio molitor* L.

Fraenkel, G. (1956). *Proc. 14th Int. Congr. Zool., Copenhagen* 1953, 383–387. Insects and plant biochemistry: the specificity of food plants for insects.

Fraenkel, G. (1958). *J. Nutr.* **65**, 361–396. The effect of zinc and potassium in the nutrition of *Tebenrio molitor* with observations on the expression of a carnitine deficiency.

Fraenkel, G. (1959). *Ann. N. Y. Acad. Sci.* **77**, 267–274. A historical and comparative survey of the dietary requirements of insects.

Fraenkel, G. and Blewett, M. (1943). *Biochem. J.* **37**, 687–692. The vitamin B complex requirements of several insects.

Fraenkel, G. and Blewett, M. (1946). *J. exp. Biol.* **22**, 172–190. Linoleic acid, Vitamin E and other fat soluble substances in the nutrition of certain insects, *Ephestia kuehniella*, *E. elutella*, *E. cautella*, and *Plodia interpunctella* (Lepidoptera).

Fraenkel, G. and Blewett, M. (1947). *Biochem. J.* **41**, 475–478. Linoleic acid and arachidonic acid in the metabolism of the insects, *Ephestia kuehniella* (Lepidoptera) and *Tenebrio molitor* (Coleoptera).

Fraenkel, G. and Friedman, S. (1957). *Vitam. & Horm.* **15**, 73–118. Carnitine.

Friend, W. G. (1958). *Annu. Rev. Ent.* **3**, 57–74. Nutritional requirements of phytophagous insects.

Friend, W. G., Backs, R. H. and Cass, L. M. (1957). *Canad. J. Res.* (*Zool.*) **35**, 535–543. Studies on amino acid requirements of the larvae of the onion maggot, *Hylemya antiqua* (Mg.), under aseptic conditions.

Friend, W. G. and Patton, R. L. (1956). *Canad. J. Res.* (*Zool.*), **34**, 152–162. Studies on vitamin requirements of larvae of the onion maggot, *Hylemya antiqua* (Mg.) under aseptic conditions.

Frings, H. and Frings, M. (1949). *Amer. Midl. Nat.* **41**, 602–658. The loci of contact chemoreceptors in insects.

Fuzeau-Braesch, S. (1959). *C. R. Soc. Biol. Paris*, **153**, 57–60. Inhibition de la pigmentation et constitution des pigments cuticulaire d'un insecte.

Gamo, T. (1941). *Bull. Seric. Silk Ind., Uyeda* **13**, 63–69. On the variation of the content of ascorbic acid during development and metamorphosis of the silkworm, *Bombyx mori.*

Gamo, T. and Seki, H. (1954). *Res. Rep. Fac. Text. Seric., Shinsku Univ.* **4,** 29–38. Some experiments on the synthetic increase of ascorbic acid content during the pupal stage of the silkworm, *Bombyx mori.*

Gilbert, L. I. and Schneiderman, H. A. (1961). *Amer. Zool.* **1,** 11–51. Some biochemical aspects of insect metamorphosis.

Gilmour, D. (1961). "The Biochemistry of Insects", 343 pp. Academic Press: London.

Giroud, A. and Ratsimamanga, R. (1936). *Bull. Soc. Chim. biol., Paris* **18,** 375–383. Distribution of vitamin C in invertebrates.

Goldsmith, T. H. (1958). *Proc. nat. Acad. Sci., Wash,* **44,** 123–126. The visual system of the honeybee.

Goldsmith, T. H. (1961). The colour vision of insects. *In* "Light and Life", (*ed. McElroy, W. D.*), pp. 771–794. John Hopkins Press: Baltimore.

Goodline, D. (1957). Unpublished notes on the feeding behaviour of the Desert locust.

Goodwin, T. W. (1949). *Biochem. J.* **45,** 472–479. Biochemistry of locusts. 2. Carotenoid distribution in solitary and gregarious phases of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) and the desert locust (*Schistocerca gregaria* Forsk).

Goodwin, T. W. (1950). *Biochem. J.* **47,** 554–562. Biochemistry of locusts. 4. Insectorubin metabolism in the desert locust (*Schistocerca gregaria* Forsk) and the African migratory locust (*Locusta migratoria migratorioides* R. & F.).

Goodwin, T. W. (1952a). *Biol. Rev.* **27,** 439–460. The biochemistry of locust pigmentation.

Goodwin, T. W. (1952b). "The Comparative Biochemistry of the Carotenoids", 356 pp. Chapman & Hall: London.

Goodwin, T. W. (1961). *Annu. Rev. Pl. Physiol.* **12,** 195–218. Biosynthesis and function of carotenoids.

Goodwin, T. W. and Srisukh, S. (1949). *Biochem. J.* **45,** 263–268. The biochemistry of locusts. 1. The carotenoids of the integument of two locust species (*Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk.).

Goodwin, T. W. and Srisukh, S. (1950). *Biochem. J.* **47,** 549–554. Biochemistry of locusts. 3. Insectorubin; the redox pigment present in the integument and eyes of the desert locust, the African locust and other insects.

Goodwin, T. W. and Srisukh, S. (1951). *Biochem. J.* **48,** 199–203. Biochemistry of locusts. 5. The green pigment of the haemolymph and integument of solitary locusts (*Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* Forsk.).

Gordon, H. T. (1959) *Ann. N. Y. Acad. Sci.* **77,** 290–351. Minimal nutritional requirements of the German roach, *Blattella germanica* L.

Grayson, J. M. and Tauber, O. E. (1943) *Iowa St. Coll. J. Sci.* **17,** 191–196. Carotin—the principal pigment responsible for variation in colouration in the adult grasshopper, *Melanoplus bivittatus* Say.

Guichard, K. M. (1955). *Anti-Locust Bull.* **21.** Habitats of the desert locust (*Schistocerca gregaria* Forskal) in Western Libya and Tibetsi.

Gunn, D. L. and Hunter-Jones, P. (1952). *Anti-Locust Bull.* **12.** Laboratory experiments on phase differences in locusts.

Hackman, R. H. (1953). *Biochem. J.* **54,** 371–377. Chemistry of insect cuticle. 3. Hardening and darkening of the cuticle.

Hamamura, Y. (1959). *Nature, Lond.* **183,** 1746–1747. Food selection by silkworm larvae.

Hamamura, Y., Hayachiya, K. and Naito, K. (1961). *Nature, Lond.* **190,** 880–881. Food selection by silkworm larvae, *Bombyx mori*. β-sitosterol as one of the biting factors.

Hamamura, Y. and Naito, K. (1961). *Nature, Lond.* **190,** 879–880. Food selection by silkworm larvae, *Bombyx mori.* Citral, linalyl acetate, linalol, and terpinyl acetate as attractants of larvae.

Hans, H. and Thorsteinson, A. J. (1961). *Ent. exp. & appl.* **4,** 165–177. The influence of physical factors and host plant odour on the induction and termination of dispersal flight in *Sitona cylindricollis* Fahr.

Haskell, P. T. (1960). Physiology and behaviour. *Report of the 7th Commonwealth Entomological Conference, July* 1960, *London.* 146–152.

Haskell, P. T., Paskin, M. W. J. and Moorhouse, J. E. (1962). *J. Insect Physiol.* **8,** 53–78. Laboratory observations on factors affecting the movements of hoppers of the desert locust.

Haydak, M. H. (1936). *Ann. ent. Soc. Amer.* **29,** 581–588. Is wax a necessary constituent of the diet of the waxmoth larva?

Haydak, M. H. and Vivino, A. E. (1943). *Arch. Biochem.* **2,** 201–207. Changes in vitamin content during the life of the worker honeybee.

Hearfield, D. A. H. and Kilby, B. A. (1958). *Nature, Lond.* **181,** 546–547. Enzymes of the tricarboxylic acid cycle and cytochrome oxidase in the fat body of the desert locust.

Heller, J., Szarkowska, L. and Michalek, H. (1960). *Nature, Lond.* **188,** 491 Ubiquinone (Coenzyme Q) in insects.

Hering, M. (1926). "Die Oekologie der blattminierenden Insektlarven", 253 pp. Gebrüder Borntraeger: Berlin.

Hering, E. M. (1950). *Naturwissenschaften,* **37,** 531–536. Monophagie und Xenophagie. Die Nahrungswahl phytophager Insekten und die geographische Herkunft ihrer Wirtspflanzen.

Hering, E. M. (1952). *Trans. 9th Int. Congr. Ent., Amsterdam* 1951, **1,** 507–513. Probleme der Cenophobie und Xenophilie bei der Wirtswahl Phytophager Insekten.

Hertz, M. and Imms, A. D. (1937). *Proc. roy. Soc.* (B) **122,** 281–297. On the responses of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) to different types of background.

Hinton, H. E. (1956). *Progr. Twent. Cent.* **174,** 292–309. Dietary requirements of insects. Amino acids and vitamins.

Hirano, C. and Ishii, S. (1957). *Bull. nat. Inst. Agric. Sci., Japan* Ser. *C.* **7,** 89-99. Nutritive values of carbohydrates for the growth of larvae of the rice stem borer, *Chilo supressalis* Walker.

Hodge, C. (1933). *Physiol. Zool.* **6,** 306–328. Growth and nutrition of *Melanoplus differentialis*. I. Growth on a satisfactory mixed diet and on diets of single plants.

Horie, Y. (1959). *Bull. Sericult. Exp. Sta. Japan* **15,** 365–382. Physiological studies on the alimentary canal of the silkworm, *Bombyx mori.* II. Carbohydrases in the digestive fluid and in the midgut tissue.

Horie, Y. (1961). *Bull. Seric. exp. Sta., Japan* **16,** 287–309. Physiological studies of the alimentary canal of the silkworm, *Bombyx mori.* III. Absorption and utilization of carbohydrates.

House, H. L. (1958). *Exp. Parasit.* **7,** 555–609. Nutritional requirements of insects associated with animal parasitism.

House, H. L. (1959). *Ann. N. Y. Acad. Sci.* **77,** 394–405. Nutrition of the parasitoid *Pseudosarcophaga affinis* (Fall). and of other insects.

House, H. L. (1961). *Annu. Rev. Ent.* **6,** 13–26. Insect nutrition.

House, H. L. (1962). *Annu. Rev. Biochem.* **31,** 653–672. Insect nutrition.

House, H. L. and Barlow, J. S. (1956). *Canad. J. Res.* (*Zool*) **34,** 182–189. Nutritional studies with *Pseudosarcophaga affinis* (Fall), a dipterous parasite of the spruce budworm, *Choristoneura fumifera* (Clem.). V. Effects of various concentrations of the amino acid mixture, dextrose, potassium ion, the salt mixture, and lard on growth and development; and a substitute for lard.

Howden, G. F. and Hunter-Jones, P. (1958). *Nature, Lond.* **182,** 1527–1528. An artificial diet for the laboratory rearing of locusts.

Howden, G. F. and Kilby, B. A. (1960). *J. Insect Physiol.* **4,** 258–269. Biochemical studies on insect haemolymph. I. Variations in reducing power with age and the effect of diet.

Howden, G. F. and Kilby, B. A. (1961). *J. Insect Physiol.* **6,** 85–95. Biochemical studies on insect haemolymph. II. The nature of the reducing material present.

Hoyle, G. (1954). *J. exp. Biol.* **31,** 260–270. Changes in the blood potassium concentration of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) during food deprivation, and the effect on neuromuscular activity.

Hunter, S. J. and Claassen, P. W. (1914). *J. econ. Ent.* **7,** 73–82. Grasshopper control in the southern division of Kansas.

Huot, L., Bernard, R. and Lemonde, A. (1957). *Canad. J. Res.* (*Zool.*) **35,** 513–518. Aspects quantitatifs des besoins en mineraux des larves de *Tribolium confusum* Duval. I. Pourcentage optimum d'un melange salin.

Huot, L., Bernard, R. and Lemonde, A. (1958). *Canad. J. Res.* (*Zool.*) **36,** 7–13. Aspects quantitatifs des besoins en mineraux del larves de *Tribolium confusem* Duval. II. Pourcentage optimum des cations Mg, Ca, Na et K.

Husain, M. A. and Ahmad, T. (1936). *Indian J. agric. Sci.* **6,** 623–664. Studies on *Schistocerca gregaria* Forsk. VI. Influence of temperature on the intensity and extent of black pattern in the desert locust hoppers bred crowded.

Hutner, S. H., Provasoli, L. and Baker, H. (1961). *Microchem. J.* Symposium Series **1,** 95–113. Development of microbiological assays for biochemical, oceanographic and clinical use.

Isely, F. B. (1938). *Ecol. Monogr.* **8,** 551–604. The relation of Texas acrididae to plants and soils.

Isely, F. B. (1941). *Ecol. Monogr.* **11,** 457–475. Researches concerning Texas Tettigoniidae.

Isely, F. B. (1944). *Ann. ent. Soc. Amer.* **37,** 47–67. Correlation between mandibular morphology and food specificity in grasshoppers.

Isely, F. B. (1946). *Ecology* **27,** 128–138. Differential feeding in relation to local distribution of grasshoppers.

Ishii, S. (1955). *Bull. nat. Inst. Agric. Sci., Japan* (C) **5,** 29–34. Note on the metabolism of steroid in the larva of *Callosobruchas chinensis* L.

Ito, T. (1960). *J. Insect Physiol.* **5,** 95–107. Effects of sugars on feeding of larvae of the silkworm, *Bombyx mori.*

Ito, T. (1961). *Bull. Seric. exp. Sta., Japan* **17,** 119–136. Nutrition of the silkworm, *Bombyx mori.* IV. Effects of ascorbic acid.

Johnston, H. B. and Buxton, D. R. (1949). *Anti-Locust Bull.* **5.** Field observations on locusts in eastern africa.

Joly, L. (1954). *C. R. Soc. Biol., Paris* **148,** 579–583. Resultats d'implantations systematiques de corpora allata à des jeunes larves de *Locusta migratoria* L.

Joly, P. (1940). *C. R. Soc. Biol., Paris* **134,** 408–410. Recherche, par voie chimique, des principles vitamines et hormones cétoniques dans le sang de reine de *Bellicositermes natalensis* (Hav.).

Joly, P. (1949). *Ann. Sci. nat. (Zool.)* **11,** 255–262. Le système endocrine retrocérébral chez les acridiens migrateurs.

Joly, P. (1951). *C. R. Soc. Biol., Paris* **145,** 1362–1364. Déterminisme endocrine de la pigmentation chez *Locusta migratoria* L.

Joly, P. (1958). *Ann. Biol. Paris* **34,** 97–118. Les corrélations humorales chez les acridiens.

Jones, B. M. and Sinclair, W. (1958). *Nature, Lond.* **181,** 926–927. Induction of melanin patterns and of hardening as separate processes in the cuticle of albino locusts with internally absorbed phenol substrates.

Kaplanis, J. N., Robbins, W. E. and Tabor, L. A. (1960). *Ann. ent. Soc. Amer.* **53,** 260–264. The utilization and metabolism of 4-C^{14}-cholesterol by the adult housefly.

Karlson, P. and Butenandt, A. (1959). *Annu. Rev. Ent.* **4,** 39–58. Pheromone (Ectohormones) in insects.

Karlson, P. and Sekeris, C. (1962). *Nature, Lond.* **195,** 183–184. N-acetyl-dopamine as sclerotizing agent of the insect cuticle.

Kennedy, J. S. (1953). *Trans. 9th Int. Congr. Ent., Amsterdam* 1951, **2,** 106–110. Host plant selection in Aphididae.

Kennedy, J. S. (1956). *Biol. Rev.* **31,** 349–370. Phase transformation in locust biology.

Kennedy, J. S. (1958). *Proc. 10th Int. Congr. Ent. Montreal* 1956, **2,** 397–404. The experimental analysis of aphid behaviour and its bearing on current theories of instinct.

Kennedy, J. S. (1961). *Symp. R. ent. Soc. Lond.* **1,** 80–90. Continuous polymorphism in locusts.

Key, K. H. L. (1950). *Quart. Rev. Biol.* **25,** 363–407. A critique on the phase theory of locusts.

Kilby, B. A. and Neville, E. (1957). *J. exp. Biol.* **34,** 276–289. Amino-acid metabolism in locust tissues.

Kreasky, J. B. (1962). *J. Insect Physiol.* **8,** 493–504. A growth factor in romaine lettuce for the grasshoppers *Melanoplus bilituratus* (Wlk.) and *M. bivittatus* (Say).

Leclercq, J. (1948a). *Arch. int. Physiol.* **55,** 412–419. Contribution a l'étude du metabolisme de l'eau chez la larve de *Tenebrio molitor* L.

Leclercq, J. (1948b). *Arch. int. Physiol.* **56,** 130–133. Aspects qualitatifs des besoins en glucides chez *Tenebrio molitor* L.

Legay, J. M. (1958). *Annu. Rev. Ent.* **3,** 75–86. Recent advances in silkworm nutrition.

Lemonde, A. and Bernard ,R. (1953). *Nat. canad.* **80,** 125–142. Aspects nutritatifs des larves de *Stegobium paniceum* L. (Anobiidae) et d'*Oryzaephilus surinamensis* L. (Cucujidae).

Lemonde, A. and Bernard, R. (1955). *Rev. canad. Biol.* **14,** 8–13. Importance de la choline et de certaines substances analogues dans la nutrition des larves de *Tribolium confusum* Duval.

Lépiney, J. de (1930). *C. R. Soc. Biol., Paris,* **104,** 265–267. Sur le comportement des larves de *Schistocerca gregaria* Forsk. Concentration et dissemination des individus, voyages des bandes larvaires, nutrition.

Levinson, Z. H. (1955). *Riv. Parassit.* **16,** 113–183. Nutritional requirements of insects.

Levinson, Z. H. (1958). *Nature, Lond.* **182,** 723–724. Fate of beta-sitosterol in housefly larvae.

Levinson, Z. H. (1962). *J. Insect Physiol.* **8,** 191–198. The function of dietary sterols in insects.

Levinson, Z. H. and Bergmann, E. D. (1957). *Biochem. J.* **65,** 254–260. Steroid utilization and fatty acid synthesis by the larva of the housefly *Musca vicina* Macq.

Lichtenstein, E. P. (1948). *Nature Lon.* **162,** 227. Growth of *Culex molestus* under sterile conditions.

Lipke, H. and Fraenkel, G. (1956). *Annu. Rev. Ent.* **1,** 17–44. Insect nutrition.

Loher, W. (1960). *Proc. roy. Soc.* (B) **153,** 380–397. The chemical acceleration of the maturation process and its hormonal control in the male of the desert locust.

Luckey, T. D. and Stone, P. C. (1960). *Science* **132,** 1891–1893. Hormology in nutrition.

Malek, S. R. A. (1957). *Nature, Lond.* **180,** 237. Schlerotization and melanization: two independent processes in the cuticle of the desert locust.

Malek, S. R. A. (1960). *Nature, Lond.* **185,** 56. Homocatechol in an insect cuticle.

Maltais, J. B. and Auclair, J. L. (1957). *Canad. Ent.* **89,** 365–370. Factors in resistance of peas to the pea aphid, *Acyrthosiphon pisum* (Harr.) (Homoptera : Aphididae). I. The sugar–nitrogen ratio.

Mason, H. S. (1955). *Advance Enzymol.* **16,** 105–184. Comparative biochemistry of the phenolase complex.

McFarlane, J. E., Neilson, B. and Ghouri, A. S. K. (1959). *Canad. J. Res.* (*Zool.*) **37,** 913–916. Artificial diets for the house cricket, *Acheta domestica* (L.).

Merton, L. H. F. (1959). *Anti-Locust Bull.* **34.** Studies on the ecology of the Moroccan locust (*Dociostaurus maroccanus* Thunberg).

Monroe, R. E., Kaplanis, J. N. and Robbins, W. E. (1961). *Ann. ent. Soc. Amer.* **54,** 537–539. Sterol storage and reproduction in the housefly.

Moussa, T. A. and Banhawy, M. (1958). *J. R. mic. Soc.* Ser. III, **78,** 111–113. On a possible relation between vitamin C and the golgi dictyosomes of insect neurones.

Nickerson, B. (1956). *Anti-Locust Bull.* **24.** Pigmentation of hoppers of the desert locust (*Schistocerca gregaria* Forskal) in relation to phase colouration.

Niemierko, W. and Wlodawar, P. (1950). *Acta Biol. exp., Varsovie* **15,** 69–76. Studies on the biochemistry of the wax moth (*Galleria mellonella*). 2. Utilization of wax constituents by the larvae.

Noland, J. L. (1954a). *Arch. Biochem. Biophys.* **48,** 370–379. Sterol metabolism in insects. I. Utilization of cholesterol derivatives by the cockroach *Blattella germanica* L.

Noland, J. L. (1954b). *Arch. Biochem. Biophys.* **52,** 323–330. Sterol metabolism in insects. II. Inhibition of cholesterol utilization by structural analogues.

Noland, J. L. and Baumann, C. A. (1951). *Ann. ent. Soc. Amer.* **44,** 184–188. Protein requirements of the cockroach, *Blattella germanica* (L.).

Norris, M. J. (1959). *Anti-Locust Bull.* **36.** Reproduction in the red locust (*Nomadacris septemfasciata Serville*) in the laboratory.

Norris, M. J. (1961). *Bull. ent. Res.* **51,** 731–753. Group effects on feeding in adult males of the desert locust *Schistocerca gregaria* (Forsk.), in relation to sexual maturation.

Okay, S. (1953). *Bull. ent. Res.* **44,** 299–315. Formation of green pigment and colour changes in the orthoptera.

Painter, R. H. (1936). *Amer. Nat.* **70,** 547–566. The food of insects and its relation to resistance of plants to insect attack.

Patton, R. L. (1953). *In* "Insect Physiology" (ed. Roeder, K. D.). Chapman & Hall: London. Excretion.

Pfadt, R. E. (1949). *Bull. Wyo. Univ.* **290.** Food plants as factors in the ecology of the lesser migratory grasshopper, *Melanoplus mexicannus* (Sauss.).

Pickford, R. (1958). *Canad. Ent.* **90,** 483–485. Observations on the reproductive potential of *Melanoplus bilituratus* (Wlk.) (Orthoptera: Acrididae) reared on different food plants in the laboratory.

Pickford, R. (1960). *Canad. Ent.* **112,** 1–10. Survival, fecundity, and population growth of *Melanoplus bilituratus* (Wlk.) (Orthoptera: Acrididae) in relation to date of hatching.

Pickford, R. (1962). *Canad. Ent.* **94,** 859–869. Development, survival and reproduction of *Melanoplus bilituratus* (Wlk.) (Orthoptera : Acrididae) reared on various food plants.

Putman, L. G. (Unpublished paper). Experiments with some native and introduced plants as foods for a *Camnula pellucida* (Scudd.) (Orthoptera: Acrididae) in western Canada.

Richards, A. G. and Brooks, M. A. (1958). *Annu. Rev. Ent.* **3,** 37–56. Internal symbiosis in insects.

Richards, O. W. and Waloff, N. (1954). *Anti-Locust Bull.* **17.** Studies on the biology and population dynamics of British grasshoppers.

Riegert, P. W. (1959). *Canada. Ent.* **91,** 35–40. The humidity reactions of grasshoppers. Humidity reactions of *Melanoplus bivitatus* (Say.) and *Camnula pellucida* (Scudd.): reactions of normal grasshoppers.

Ritchot, C. and McFarlane, J. E. (1961). *Canad. J. Res.* (*Zool.*) **39,** 11–15. The B vitamin requirements of the house cricket.

Robbins, W. E., Dukty, R. C., Monroe, R. E. and Kaplanis, J. N. (1962). *Ann. ent. Soc. Amer.* **55,** 102–104. The metabolism of H^3-β-sitosterol by the German cockroach.

Robbins, W. E., Kaplanis, J. N., Monroe, R. E. and Tabor, L. A. (1961). *Ann. ent. Soc. Amer.* **54,** 165–168. The utilization of dietary cholesterol by German cockroaches.

Robbins, W. E. and Shortino, T. J. (1962). *Nature, Lond.* **194,** 502–503. Effect of cholesterol in the larval diet on ovarian development in the adult housefly.

Rockstein, M. (1957). *Annu. Rev. Ent.* **2,** 19–36. Some aspects of intermediary metabolism of carbohydrates in insects.

Roonwal, M. L. (1953). *J. zool. Soc. India* **5,** 44–58. Food preference experiments with the desert locust, *Schistocerca gregaria* (Forskal) in its permanent breeding grounds in the Mekran (S. Baluchistan).

Roth, L. M. and Eisner, T. (1962). *Annu. Rev. Ent.* **7,** 107–136. Chemical defences of arthropods.

Roussel, P. G. (1958). *J. N.Y. ent. Soc.* **66,** 49–58. Effects of various factors on the synthesis of ascorbic acid by the American cockroach *Periplaneta americana* L.

Sang, J. H. (1956). *J. exp. Biol.* **33,** 45–72. The quantitative nutritional requirements of *Drosophila melanogaster.*

Sarma, P. S. and Bhagvat, K. (1942). *Curr. Sci.* **11,** 394. The synthesis of vitamin C by rice moth larvae (*Corcyra cephalonica* Staint.).

Schmialek, P. (1961). *Z. Naturf.* **16b,** 461. Die Identifizierung zweier im Tenebriokot und im Hefe vorkommender Substanzen mit Juvenilhormonwirkung.

Singh, Asket, (1957). *Res. Bull. Panjab Univ. Hoshiarpur* (*Zool.*) **108,** 291–298. Some critical observations on the feeding activity of the desert locust, *Schistocerca gregaria* (Forskal), under different environmental conditions.

Singh, K. R. P. and Brown, A. W. A. (1957). *J. Insect. Physiol.* **1,** 199–220. Nutritional requirements of *Aedes aegypti L.*

Skoog, F. E., Cowan, F. T. and Connin, R. V. (1960). *U.S.D.A. Agr. Res. Service Special Report Z*–**109.** Laboratory tests with insecticides, attractants, and repellants, Bozeman, Montana, 1959–60.

Slifer, E. H. (1954). *Proc. R. Ent. Soc. Lond.* (A) **29,** 177–179. The reaction of a grasshopper to odorous material held near one of its feet (Orthoptera: Acrididae).

Slifer, E. H. (1955). *J. exp. Zool.* **130,** 301–317. The detection of odours and water vapour by grasshoppers (Orthoptera: Acrididae) and some new evidence concerning the sense organs which may be involved.

Slifer, E. H. (1956). *Proc. R. ent. Soc. Lond.* (A) **31,** 95–98. The response of a grasshopper, *Romalea microptera* (Beauvois), to strong odours following amputation of the metathoracic leg at different levels.

Smith, D. S. (1959). *Ann. ent. Soc. Amer.* **52,** 674–680. Utilization of food plants by the migratory grasshopper Melanoplus bilituratus (Walker) (Orthoptera: Acrididae), with some observations on the nutritional value of the plants.

Smith, D. S. (1960). *Canad. Ent.* **92,** 103–107. Effects of changing phosphorus content of the food plant on the migratory grasshopper *Melanoplus bilituratus* (Walker) (Orthoptera: Acrididae).

Smith, D. S., Handford, R. H. and Chefurka, W. (1952). *Canad. Ent.* **84,** 113–117. Some effects of various food plants on *Melanoplus mexicanus mexicanus* Sauss.) (Orthoptera: Acrididae).

Smith, D. S. and Northcott, F. E. (1951). *Canad. J. Res.* (*Zool.*) **29,** 297–304. The effects on the grasshopper, *Melanoplus mexicanus mexicanus* (Sauss.) of varying the nitrogen content in its food plant.

Stower, W. J. (1959). *Anti-Locust Bull.* **32.** The colour patterns of hoppers of the desert locust (*Schistocerca gregaria* Forskal).

Stower, W. J., Davies, D. E. and Jones, I. B. (1960). *J. anim. Ecol.* **29,** 309–339. Morphometric studies of the desert locust *Schistocerca gregaria* (Forsk.).

Strange, P. H. (1961). *J. exp. Biol.* **38,** 237–248. The spectral sensivity of *Calliphora* maggots.

Strangways-Dixon, J. (1961a). *J. exp. Biol.* **38,** 225–235. The relationship between nutrition, hormones and reproduction in the blowfly *Calliphora erythrocephala* (Meig.) I. Selective feeding in relation to the reproduction cycle, the corpus allatum volume and fertilization.

Strangways-Dixon, J. (1961b). *J. exp. Biol.* **38,** 637–646. The relationship between nutrition, hormones and reproduction in the blowfly *Calliphora erythrocephala*

(Meig.). II. The effect of removing the ovaries, the corpus allatum and the median neurosecretory cells upon selective feeding, and the demonstration of corpus allatum cycle.

Sussman, A. S. (1949). *Quart. Rev. Biol.* **24,** 328–341. The function of tyrosinase in insects.

Tauber, O. E., Drake, C. J. and Decker, G. C. (1945). *Iowa St. Coll. J. Sci.* **19,** 343–359. Effects of different food plants on egg production and adult survival of the grasshopper, *Melanoplus bivittatus* Say.

Thorpe, W. H., Crombie, A. C., Hill, R. and Darragh, J. H. (1947). *J. exp. Biol.* **23,** 234–266. The behaviour of wireworms in response to chemical stimulation.

Thorsteinson, A. J. (1953a). *Canad. J. Res.* (*Zool.*) **31,** 52–72. The chemotactic responses that determine host specificity in an oligophagous insect (*Plutella maculipennis* (Curt.) (Lepidoptera).

Thorsteinson, A. J. (1953b). *Canad. Ent.* **85.** 276–282. The role of host selection in the ecology of phytophagous insects.

Thorsteinson, A. J. (1958a). *Proc. 10th Int. Congr. Ent. Montreal* 1956 **2,** 599–602. Acceptability of plants for phytophagous insects.

Thorsteinson, A. J. (1958b). *Ent. exp. & appl.* **1,** 23–27. The chemotactic influence of plant constituents on feeding by phytophagous insects.

Thorsteinson, A. J. (1960). *Annu. Rev. Ent.* **5,** 193–218. Host selection in phytophagous insects.

Tietz, A. (1961). *J. Lipid Res.* **2,** 182–187. Fat synthesis in cell-free preparations of the locust fat-body.

Trager, W. (1953). *In* "Insect Physiology" (ed. Roeder, K. D.), pp. 350–386. Chapman & Hall: London. Nutrition.

Treherne, J. E. (1958a). *J. exp. Biol.* **35,** 297–306. The absorption of glucose from the alimentary canal of the locust *Schistocerca gregaria* (Forsk.).

Treherne, J. E. (1958b). *J. exp. Biol.* **35,** 611–625. The absorption and metabolism of some sugars in the locust, *Schistocerca gregaria* (Forsk.).

Uvarov, B. P. (1928). "Locusts and Grasshoppers. A Handbook for their Study and Control", 352 pp. Imperial Bureau of Entomology: London.

Uvarov, B. P. (1948). *Trans. R. ent. Soc. Lond.* **99,** 1–75. Recent advances in acridology; anatomy and physiology of Acrididae.

Uvarov, B. P. (1961). *Proc. R. ent. Soc. Lond.* (C) **25,** 52–58. Quantity and quality in insect populations.

Van der Kloot, W. G. (1960). *Annu. Rev. Ent.* **5,** 35–52. Neurosecretion in insects.

Vanderzant, E. S. (1957). *J. econ. Ent.* **50,** 219–221. Growth and reproduction of the pink bollworm on an amino acid medium.

Vanderzant, E. S. (1958). *J. econ. Ent.* **51,** 309–311. The amino acid requirements of the pink bollworm.

Vanderzant, E. S. (1959). *J. econ. Ent.* **52,** 1018–1919. Inositol; An indispensable dietary requirement for the boll weevil.

Vanderzant, E. S., Kerur, D. and Reiser, R. (1957). *J. econ. Ent.* **50,** 606–608. The role of dietary fatty acids in the development of the pink bollworm.

Vanderzant, E. S., Pool, M. C. and Richardson, C. D. (1962). *J. Insect Physiol.* **8,** 287–297. The role of ascorbic acid in the nutrition of three cotton insects.

Volkonsky, M. (1942). *Arch. Inst. Pasteur Algér.* **20,** 236–248. Observations sur le comportement du criquet pèlerin ((*Schistocerca gregaria* Forsk.) dans le sahara Algéro-nigérien.

Waldbauer, G. P. and Fraenkel, G. (1961). *Ann. ent. Soc. Amer.* **54,** 477–485. Feeding on normally rejected plants by maxillectomised larvae of the tobacco hornworm, *Protoparce sexta* (Lepidoptera Sphingidae).

Wallace, G. K. (1958). *J. exp. Biol.* **35,** 765–775. Some experiments on form perception in the nymphs of the desert locust, *Schistocerca gregaria* Forskal.

Watanabe, T. (1958). *Nature, Lond.* **182,** 325–326. Substances in mulberry leaves which attract silkworm larvae (*Bombyx mori*).

Watson, J. R. and Bratley, H. E. (1940). *Florida Ent.* **23,** 7–10. Preliminary report on lubberly locust control.

Weis-Fogh, T. (1952). *Phil. Trans.* (B) **237,** 1–36. Fat combustion and metabolic rate of flying locusts (*Schistocerca gregaria* Forskal).

Wigglesworth, V. B. (1961). *J. Ins. Physiol.* **7,** 73–78. Some observations on the juvenile hormone effect of farnesol in *Rhodnius prolixus* Stal (Hemiptera).

Wilde, J. de and Stegwee, D. (1958). *Arch. neerl. Zool.* (*Supplement* 1) **13,** 277–289. Two major effects of the corpus allatum in the adult colorado beetle.

Williams, L. H. (1954). *Trans. R. ent. Soc. Lond.* **105,** 423–454. The feeding habits and food preferences of Acrididae and the factors which determine them.

Wollman, E., Giroud, A. and Ratsimamanga, R. (1937). *C. R. Soc. Biol., Paris* **124,** 434–435. Synthèse de la vitamine C chez un insecte orthoptère (*Blatella germanica*) en élevage aseptique.

Yokoyama, T. (1963). *Annu. Rev. Ent.* **8,** in the press. Sericulture.

Young, R. G. (1961). *Ann. ent. Soc. Amer.* **54,** 657–659. The effects of dietary beeswax and wax components on the larvae of the greater wax moth, *Galleria mellonella* (L.).

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The Biochemistry of the Insect Fat Body

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I. Introduction

Until a few years ago much of the knowledge of the biochemistry of insects was derived from experiments in which homogenates of whole insects were used. While such experiments can give useful general information concerning the occurrence, quantity and interconversion of metabolites and the enzyme systems involved, they reveal little about location or transfer within the insect. Recent refinements of biochemical techniques have permitted an increasing amount of work on individual tissues, so that the results of insect biochemists are now comparable to those of mammalian biochemists who do not do their experiments on homogenates of whole rats. There is no reason to suspect that the intermediary metabolism of insects is any less complex than that of higher animals, and the question arises where in the insect much of this metabolism takes place. Where, for instance, is blood protein and blood trehalose synthesized, or dietary carbohydrates converted into fats, or purines and pteridines formed? During recent years increasing attention has been paid to the possibility that the insect fat body may be a site of intermediary metabolism, as distinct from its more passive role of serving as a depot for the storage of fats, protein and carbohydrate reserves. A recurrent suggestion is that the fat body may in some respects be analogous to the mammalian liver.

Biochemical studies on the fat body have now reached a stage at which a review is both possible and desirable. The literature is widely scattered, and several difficulties are encountered in trying to make a coherent account. One is the wide range of different insect species that have been used; often the fragmentary results of different workers have to be brought together in order to draw up a possible scheme for a generalized fat body. Now this could be misleading if the biochemistry of fat bodies of different insects varied appreciably, although there is no reason at present to suspect that this is so. Another difficulty arises from the nature of the life cycle of the insect. In the different stages such as the immature forms, prepupa, diapausing and developing pupae and the adult form, the biochemical processes taking place in the fat body may vary according to the specific point in the insect's development. Some general conclusions published in the literature might well be different if insects of another age had been used. For instance, the enzyme *isocitric lyase* is absent from mature sixth instar *Prodenia* larvae but present in prepupae, so that unless two workers specified the age of their material, opposite conclusions might be drawn about the occurrence of the glyoxalate cycle in *Prodenia*. Some biochemical pathways have been worked out in detail using tissues

such as insect muscle or gut, and a few cursory experiments with fat body tissue have sometimes suggested that similar pathways are probably operative in this tissue. The reviewer has tried to avoid generalizing about the fat body on the basis of analogy with other tissues, otherwise his account would have tended to develop into a broad survey of insect biochemistry. Similarly, it was not felt appropriate to deal, other than briefly, with specialized topics, such as insect endocrinology, biochemical genetics or the phenomenon of intracellular symbionts, since such matters would best be considered in a much wider context, in which the participation of the fat body is but one facet.

A general account of the biochemistry of insects has been given by Gilmour (1961) and some aspects of intermediary metabolism in the fat body are considered briefly in a review by Urich (1961).

II. Nature of the Fat Body

When an insect is dissected the fat body is seen as one of the most conspicuous organs, especially in larval forms, where it sometimes occupies the larger part of the body cavity. It may be glistening, white and opaque, as in *Calliphora* larvae, or show various shades of yellow or light green depending on the species; it is, for example, deep yellow in *Schistocerca*. The cells that make it up are usually arranged in lobes or sheets in a manner which is more or less constant for a given species, although a wide variety of arrangements are found. Buys (1924) studied larval fat bodies in a representative series of insects from different orders and the forms she described included irregular lobulated masses, thin leaf-like sheets, anastomosing strands, ribbons, much fenestrated sheets, etc. In *Aleurodes* the cells are not joined but float freely in the haemolymph. Frequently two portions of fat body can be distinguished, one which is peripheral and is attached to the over-lying epidermis, and a more central portion around the gut. The ribbon or sheet-like structure is such as to expose a maximum area to the blood which is in contact everywhere with the fat body, so that this organ would be well suited for the rapid exchange of metabolites with the blood, while oxidative processes could be facilitated by the finely branched tracheoles which penetrate to all parts of the tissue.

The fat body cells proper, the *trophocytes*, increase in size during post-embryonic periods of feeding, becoming filled with reserves of fat, glycogen and protein so that finally they become some of the largest cells in the insect body and take up polygonal shapes because of their close packing. Intracellular bacteria occur in many insects and are usually confined to certain cells, the *mycetocytes*, which may be associated with the gut, gonads, fat body, etc., depending on the species. The fat body mycetocytes

in *Blatta*, cells packed with rod-like bacteria, are clearly shown in electron microscope photographs published by Meyer and Frank (1957). In a few cases, certain cells of the fat body contain granules of pigment, and these cells, termed *chromatocytes*, may contribute to the colour pattern of the insect, as described by Hinton (1958) for *Thaumalea* larvae.

Large cells, called *oenocytes*, occur in almost all insects, originating in the embryo from the ectoderm and become distributed in various ways in different insects. Sometimes they remain associated with the epidermis, or form groups in various parts of the body or quite frequently are distributed as free cells scattered throughout the fat body. Their function is, as A. G. Richards has observed, more suspected than known. It is generally believed that they may be concerned in intermediary metabolism. They undergo a cycle of changes during moulting and may be concerned with the formation and secretion of lipoprotein required for new epicuticle (Wigglesworth, 1959). The close association of oenocytes with the fat body means that much of the work with fat body has involved the use of tissue "contaminated" with oenocytes, and the possibility must be kept in mind that their presence may have contributed to the results observed. This risk could be reduced by careful choice of tissue; for example, Coupland (1957) has shown that while oenocytes are numerous in the peripheral parts of *Schistocerca* fat body, they are only occasionally observed in fat body from the central region.

III. Carbohydrates and their Metabolism

A. Glycogen

Glycogen forms an important carbohydrate reserve in the insect. Its distribution in adult *Drosophila melanogaster* was examined by Wigglesworth (1949), who found the bulk of the glycogen in the abdominal fat body as large peripheral intracellular deposits. Other sites, in order of decreasing importance, were the halteres, flight muscle, proventriculus and the mid-gut cells. The amount of glycogen in insect fat bodies can vary widely, depending on the stage in the life history, on the past nutritional level and on the demands made by energy requirements.

1. *Variation with age*

In *Tenebrio molitor*, for instance, the maximum accumulation of glycogen occurs at the end of the larval stage, disappears during pupation but reappears at the end. Much of the glycogen may be utilized during metamorphosis; for example, Shigematsu (1956) found that the glycogen content of the fat body of the silkworm was 52·5 mg/g dry weight at the

beginning of the fifth instar, rising to 215 mg/g at the end, but had fallen to 35·6 mg/g by the second day of the pupal stage. In well-nourished insects the glycogen content of the fat body is quite high, making up, for instance, 17–27% of the dry weight in mature larvae of *Prodenia eridania*, 17–21% in silkworms, while in mature bee larvae, in which the fat body largely fills the body cavity and makes up 65% of the body weight, no less than 33% of the dry weight of the entire insect consists of glycogen.

2. *Variation with nutritional level*

A study of the depletion of the reserves in the fat body during starvation, and their replacement when various foods were supplied, was made by Wigglesworth (1942) with the larva of the yellow fever mosquito, *Aëdes aegypti*. This is a most convenient experimental animal for the purpose since the fat body can be observed under the microscope in the living larva using transmitted light. The inclusions in a given cell can therefore be followed from hour to hour or day to day, and the tissue fixed and stained at a desired stage. The distribution of glycogen could be seen very simply by immersing the living larva in 0·05% iodine solution. There is a plentiful supply of glycogen, fat and protein in the fat body of the newly emerged fourth instar larva, but during starvation these reserves are all used up concurrently, so that after 10–15 days, glycogen and fat are almost undetectable. The efficiency of different sugars as foodstuffs was tested by Wigglesworth by placing the completely starved larvae in dilute sugar solutions. Glucose was the most effective in replenishing the reserve glycogen which was laid down in the fat body within 6 h: abundant deposits were formed after 12 h. Fructose, sucrose and maltose were utilized in a similar manner although rather more slowly, while other sugars such as trehalose, mannose and lactose gave glycogen deposits in the gut but not in the fat body within 12 h. Some sugars such as sorbose, arabinose and raffinose gave no glycogen in any site, and were presumably either not absorbed or metabolized, or only very slowly. Casein, alanine and glutamic acid gave rise to fat body glycogen, while olive oil gave fat deposits but no glycogen. The fat body is thus seen to be an organ which can readily synthesize glycogen from the commoner sugars and glucogenic amino acids but not from fat, as might be expected. Adult *Drosophila* show a similar concurrent reduction of glycogen and fat reserves of the fat body during starvation (Wigglesworth, 1949).

3. *Effect of exercise*

There is now good evidence for believing that the energy required for continuous flight by *Diptera* is derived almost exclusively from the aerobic

metabolism of carbohydrate, although other insects (locusts and butterflies), have been shown to utilize fat. Chadwick (1947) found that the respiratory quotient for *Drosophila* in flight was essentially 1·0, indicative of carbohydrate metabolism, while Wigglesworth (1949) showed that, by feeding sugars, *Drosophila* flown to exhaustion could be revived rapidly so that flight was resumed. Glucose was very efficient, requiring only 30–40 sec, sucrose, mannose and trehalose took 1–1½ min and fructose 2–3 min. Exhausted *Phormia regina* can likewise resume flight within 30 sec of being fed with glucose (Hudson, 1958). Until a few years ago it would have been assumed that the muscles were utilizing glucose derived from the breakdown of muscle glycogen or were using blood glucose, the level of which could be maintained by the breakdown of fat body glycogen. This scheme has been modified since the discovery of trehalose in insects (see below), but one would still expect the same overall effect, i.e. sustained flight should lead to a fall in the fat body reserves of glycogen, and this has been confirmed in several species. Using histochemical methods, Wigglesworth (1949) demonstrated the depletion of glycogen reserves in the fat body of *Drosophila* after flight to exhaustion, and Clements (1955) obtained similar results using the mosquito, *Culex pipiens*. In both species there was no apparent reduction in the fat reserves during flight. Quantitative measurements on the changes in the total body glycogen produced by sustained flight have been made by Williams *et al.* (1943) using *Drosophila funebris* and *Lucilia sericata*, and by Hudson (1958) using *Phormia regina*, and in each case a progressive fall in glycogen was found during flight. With *Phormia*, for example, there was a steady fall for the first 45 min, when about 32% of the initial glycogen remained (as estimated from controls), while after 3 h flight, only 5% of the body glycogen remained. In *Drosophila*, the glycogen reserves are greatly reduced but not exhausted when flight ceases, and some remain in the fat body and in the halteres, while those in the oocytes in the female are largely untouched. The extent of the depletion depends on the age of the fly: it is more complete with young flies (5–7 days old) than with older ones (4 weeks old) suggesting a more efficient mobilization of reserves in young flies while in older ones the rate of mobilization may be slower and less able to meet the energy demands of the flight muscles, leading to earlier exhaustion and cessation of flight.

4. *Biosynthesis of glycogen*

It appears probable that both the phosphorylase and the UDPG routes for glycogen synthesis are operative in the fat body. Trivelloni (1960) has shown that incubation of fat body preparations from *Schistocerca can-*

cellata with uridine diphosphate glucose-^{14}C (UDPG) resulted in the transfer of a part of the radioactivity to the glycogen, and when this was treated with β-amylase, all the radioactivity was liberated as maltose. There was thus enzymic transfer of glucose residues from the UDPG to the ends of the glycogen primer chain by the formation of $\alpha(1\rightarrow4)$ linkages. Incubation of fat body extracts with labelled glucose-1-phosphate also led to some incorporation of radioactivity into glycogen so that some synthesis of glycogen could be taking place under the action of phosphorylase.

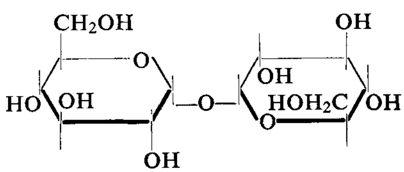
B. GLUCOSE

Assays of reducing power in insect blood by the conventional methods usually give values comparable with those for mammalian blood when expressed as glucose, but Hemmingsen in 1924 found that only one-quarter of the reducing material in *Bombyx* was fermentable by yeast, and this is generally true for other insect species. The true reducing sugar has been identified as glucose, although fructose may occur exceptionally, as in adult honey bees and in *Gastrophilus intestinalis* larvae (Levenbook, 1950). The levels of reducing sugar in blood usually range in different species between zero and about 30 mg/100 ml, which, as Wyatt and Kalf (1957) remarked, appeared remarkably low for such metabolically active animals as insects with a high dietary intake of sugars. The discovery that reducing sugar may represent only 5% of the total blood sugar has resolved this paradox.

C. TREHALOSE

1. *Discovery*

Wyatt and Kalf (1956) found that the major carbohydrate component of insect blood is the non-reducing disaccharide trehalose (I), a compound previously mainly associated with fungi, although it had indeed first been isolated by Berthelot in 1859 from Trehala manna, a secretion of the weevil, *Larinus nidificans*.



I. Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside)

Wyatt and Kalf isolated a crystalline specimen of trehalose from the blood of pupae of the silk moth, *Telea polyphemus*, and characterized it

fully, and they also showed the presence of trehalose by chromatography in all of the nine other insect species (from five orders) that they examined. Shortly afterwards, crystalline trehalose was isolated by Howden and Kilby (1956, 1961) from the blood of the Desert Locust, *Schistocerca gregaria*, and it now appears probable that this sugar is a universal constituent of insect bloods, and it has also been shown to occur in many helminths and other invertebrata from most of the major phyla (Fairbairn, 1958). The amounts of trehalose in insect bloods are often surprisingly high: for instance, up to 1500 mg/100 ml in *Schistocerca*, about 2000 mg/100 ml in *Hyalophora cecropia*, while the present record appears to be held by the larva of a solitary bee, *Anthopera* sp, in which Duchateau and Florkin (1959) found 6554 mg/100 ml blood.

As trehalose is a non-reducing sugar, it is not detected by the usual chemical methods for assay of blood sugar, but if it is subjected to hydrolysis by heating with dilute acid, the α-α bond is split, so that one molecule gives rise to two molecules of glucose. This explains the observation of Kuwana in 1937 that much reducing material was produced by the acid hydrolysis of silkworm blood.

2. *Formation from absorbed sugars*

Treherne (1958a, b) has shown that ^{14}C-glucose introduced into the gut of the locust is rapidly absorbed and is almost completely converted into trehalose which accumulates in the blood. The absorption was largely confined to the caeca, the proventriculus being less active and there was no significant uptake from the hind-gut. The results of an extensive investigation were consistent with the hypothesis that absorption occurs by a diffusion process across the gut wall, rather than by some active transporting system of the type believed to occur in sugar absorption across the mammalian gut wall. The glucose concentration in locust blood is low (around 24 mg/100 ml) and is kept down by the rapid conversion of absorbed sugar into trehalose by the fat body. The percentage absorption of glucose was shown to be the same when 0·002 M and 0·02 M glucose was placed in the gut, and a low blood glucose level was maintained, but if 0·2 M glucose was used, it appeared that the fat body was unable to convert glucose into trehalose at a sufficiently rapid rate to keep up with the glucose diffusing in, so that glucose accumulated in the blood, reducing the concentration gradient across the gut wall and so tending to reduce the percentage absorption of the glucose at the highest dosage level. If, on the other hand, the concentration of the glucose in the caeca was very low and comparable with that in the blood and there was thus no effective concentration gradient across the gut wall, no net absorption was ob-

served, although some exchange of labelled glucose across the gut wall did occur. Additional evidence for the hypothesis was given by experiments in which it was shown that the rate at which sugar passed through the gut wall was the same *in vivo* as *in vitro* when the gut was suspended in a relatively large volume of saline containing iodoacetate and cyanide, poisons which would almost certainly inhibit any active transport system. In one case the gradient was maintained by the removal of glucose by its conversion into trehalose, and in the other by dilution by the saline. Labelled fructose and mannose were also absorbed from the gut *in vivo* and gave rise to some labelled trehalose, but the percentages absorbed in a given time were, in general, less than with glucose, and the conversion to trehalose slower, so that these sugars were also present in the blood in appreciable amounts.

3. *Site of trehalose formation*

The work of Treherne did not yield any positive evidence as to the site of conversion of glucose or other simple sugars into trehalose in the insect. His observation that the percentage conversion of glucose into trehalose was almost identical in experiments in which the glucose was absorbed through the gut wall or injected directly into the blood, suggested that the conversion did not occur during the passage of the sugar through the gut wall. Candy and Kilby (1959, 1961) incubated ^{14}C-glucose with intact fat body tissue from *Schistocerca* and observed appreciable formation of labelled trehalose, but if locust leg muscle, blood, crop, mid- or hind-gut was used, no comparable conversion was obtained, and they concluded that the fat body was the main site of trehalose biosynthesis from glucose. Confirmatory evidence was obtained by Clegg and Evans (1961) working with the blowfly, *Phormia regina*, when intact fat body was found to be highly active, while blood, mid-gut and flight muscle were inactive. Now that the main site of trehalose formation has been identified, the matters of particular interest are the nature of the biochemical pathway of synthesis, the source of the substrates utilized, and the quantitative aspects of the rates of trehalose formation and utilization, the latter requiring some knowledge of the function of this sugar in the insect economy. Some information is now available on all of these topics.

4. *Pathway of biosynthesis*

A cell-free extract prepared from the fat body of *Schistocerca* and fortified with adenosine triphosphate (ATP) and uridine diphosphate glucose (UDPG) was shown by Candy and Kilby (1961) to be able to convert ^{14}C-glucose into ^{14}C-trehalose. The intermediates in trehalose formation

were identified by autoradiography of chromatograms of material taken at different times from the incubation mixture. The glucose largely disappeared within 15 min, giving rise to glucose-6-phosphate, which reacted with UDPG to produce trehalose phosphate. A specific phosphatase then hydrolysed this to trehalose. The fat body also contained the enzymes required for the regeneration of the UDPG. The scheme is shown in Fig. 1, and involves the six enzymes named. A similar pathway is known to be operative in yeast (Cabib and Leloir, 1958), so that it seems probable that insects and fungi make trehalose in the same manner.

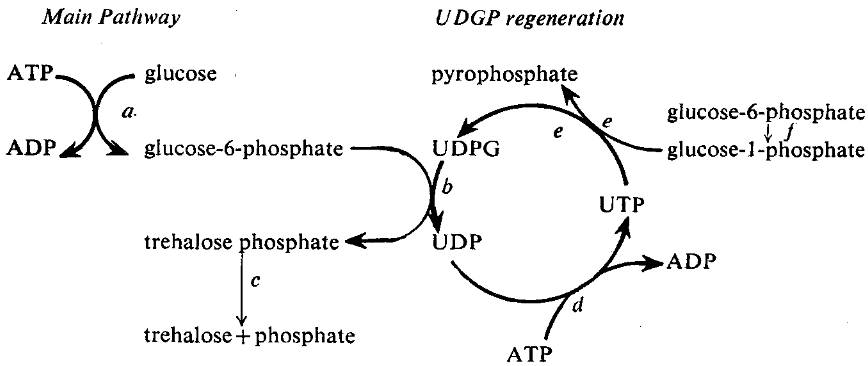


FIG. 1. Scheme for trehalose biosynthesis in Locust fat body. Enzymes involve (a) hexokinase; (b) phosphotrehalose uridine diphosphate transglucosidase; (c) trehalose-6-phosphatase; (d) nucleoside diphosphokinase; (e) UDPG pyrophosphorylase; (f) phosphoglucomutase.

Trehalose-6-phosphatase has been purified about sixtyfold by Friedman (1960a) who started with a homogenate of whole flies (*Phormia regina*) and used a diethylaminoethylcellulose (DEAE) column. The purified enzyme showed a pH optimum at 7·0 and required divalent ions for activity, Mg^{++} being the most effective. It showed a high specificity, readily splitting trehalose-6-phosphate, but was inactive towards a range of monosaccharide and nucleoside phosphates. Disaccharide phosphates other than trehalose-6-phosphate were not available for test however. Candy and Kilby showed that the enzyme was present in *Schistocerca* fat body, and Friedman demonstrated it in *Phormia* blood. The conversion of glucose into trehalose occurs rapidly, as shown by experiments by Clegg and Evans (1961) in which adult *Phormia* were injected with radioactive glucose, and 2% of the activity was found in the blood trehalose within 30 sec, rising to 50% within 2 min and to 85% in 5 min. Labelled glucose,

mannose and fructose were readily utilized by intact fat body when incubated *in vitro*, to give trehalose of high specific activity, so that there is no doubt of the ability of the fat body to absorb exogenous sugars from the environment and convert them into trehalose. The same workers also showed that although *Phormia* fat body contains less than 25 μg/mg dry weight fat body of trehalose, nevertheless, when it is incubated in saline, trehalose is liberated into the medium at a rate of about 250 μg/mg dry weight fat body/h indicating synthesis from endogenous sources, such as fat body glycogen.

5. *Energy expenditure and trehalose synthesis*

Clegg and Evans (1961) have shown that as *Phormia* is flown to exhaustion, the rate of energy expenditure, as indicated by the wing beat frequency, falls steadily and at the same time, the trehalose level in the blood also falls steadily. Injection of trehalose into exhausted flies led to resumed flight, and the increase in wing beat frequency over that at exhaustion was directly related to the amount of trehalose injected. These investigators concluded that the concentration of trehalose in the blood determines (at least in part) the rate of energy expenditure by flight muscles. The trehalose level in the blood is determined by the relative rates of two processes—the removal of trehalose by utilization and its synthesis and replacement by the fat body. It has been estimated that *Phormia* expends carbohydrate during flight at the rate of 15 μg/min. The total amount of trehalose in the blood (not more than 200 μg) would only support flight for a few minutes unless replaced. Since sustained flight can last for 2 to 3 h, it is clear that the fat body is synthesizing and secreting trehalose at a rate almost equal to the rate of utilization. The small deficit leads to a slow fall in trehalose concentration, until after 2–3 h, the concentration has fallen to about one-quarter of the initial value, and it appears that at this level trehalose cannot be supplied to the wing muscles at a sufficient rate, so the wings cease beating and the fly is "exhausted" although there is still some trehalose in the blood and the glucose level has remained almost unchanged.

6. *The breakdown of trehalose*

Frerejacque (1941) examined insects which lived on fungi, in the expectation that their digestive tracts would secrete an enzyme capable of breaking down the trehalose which is widely distributed among cryptograms, and a trehalase was indeed found in homogenates of these insects. When his survey was widened, he found, rather surprisingly, that all other insects that he examined also contained trehalase, although they fed on

higher plants in which this sugar is absent. He therefore came close to discovering trehalose in insects, but unfortunately apparently overlooked the possibility that the substrate might originate in the insect itself rather than exclusively in the diet. In 1956, Howden and Kilby showed that the locust fat body and blood contained the enzyme, which is thus not simply a digestive one.

The properties of the enzyme were examined in greater detail by Kalf and Rieder (1958) who achieved a fifty-fold purification starting with homogenates of the larval stage of the wax moth, *Galleria mellonella*. The enzyme appears to be a specific trehalase as none of the seventeen other sugars and glucosides tested were hydrolysed, and the purified insect enzyme has been suggested as a highly specific tool for the assay of trehalose. It is a stable enzyme, with a pH optimum of 5·5 and hydrolyses the substrate to two molecules of glucose, the reaction going effectively to completion ($K_m = 1{\cdot}3 \times 10^{-4}$M).

Trehalase has also been isolated from *Phormia regina* by Friedman (1960b) and a thousandfold purification achieved by using a DEAE column. The properties were very similar to those of the enzyme prepared by Kalf and Rieder from *Galleria*—a pH optimum of 5·6 and a $K_m = 6{\cdot}7 \times 10^{-4}$ M were recorded.

Frerejacque had reported a stimulation of activity by phosphate, which suggested the possibility of a phosphorolytic cleavage, but this has not been confirmed and the hydrolytic reaction is now known to be operative. There is little information about the distribution of trehalase among different tissues in the insect. It certainly occurs in the fat body, since Howden and Kilby (1956) found that a phosphate buffer extract of an acetone-dried locust fat body homogenate would produce glucose from trehalose at the rate of 6200 μg glucose/h per g acetone-dried homogenate, but it was necessary to add fluoride to prevent loss of glucose by glycolysis. Zebe and McShan (1959b) reported a rather low activity in the fat body of the woodroach, *Leucophaea maderae*. Insect muscle is active, the enzyme occurring in the flight muscle of *Phormia* (Friedman, 1960b) and the thoracic muscle of the woodroach. Results with insect bloods are more variable.

Blood from the locust produced 1700 μg glucose/h per g blood from trehalose as substrate, but no trehalase activity was found in experiments using woodroach blood or silkworm blood. It is possible that these contradictory results may be due to the experimental conditions employed, since Friedman (1961) has made the interesting observation that while undiluted *Phormia* blood is inactive and no changes in the concentrations of glucose and trehalose occur on incubation, trehalase activity appears

and reaches its maximum value when the blood has been diluted one hundred times. The activity of a purified trehalase was reduced to a third of its value by addition of dialysed blood, but unaffected by the addition of dialysed blood which had been heated to 80° for 3 min. More gentle heat treatment of blood reduced the degree of dilution which was required before the endogenous trehalase activity could manifest itself. Evidence at present suggests the presence of an inhibitory compound of high molecular weight and less stable to heat treatment than trehalase. The biochemical process which releases trehalase from inhibition *in vivo* is obscure, as are the physiological conditions which set the releasing mechanism in action. It would be interesting to know if the inhibitor also occurs in the fat body and, through its action on trehalase, contributes towards the regulation of the net amount of trehalose produced there.

A pure mitochondrial preparation from thoracic muscle of the woodroach was shown by Zebe and McShan (1959b) to have a very high trehalase activity, but there is no similar information available about fat body mitochondria.

D. GLUCOSIDES

In addition to its role in the biosynthesis of trehalose and glycogen already mentioned, uridine diphosphate glucose is also used for the synthesis of glucosides in the fat body.

Myers and Smith (1954) were the first to show that if hydroquinone or m-aminophenol were injected into *Locusta migratoria*, the corresponding β-glucosides could be detected in the excreta. Information about the site of glucoside formation was given by Trivelloni in 1960, who found that the glucoside of hydroquinone (arbutin) could be detected chromatographically in the fat body of *Schistocerca cancellata* after injection of hydroquinone into the insect. Its formation *in vitro* could be demonstrated by incubation of homogenized fat body with hydroquinone and UDPG, but it was not formed if the UDPG was omitted or replaced by glucose-1-phosphate. Smith and Turbert (1961) found that intact fat body from *Schistocerca gregaria* would convert 4-methylumbelliferone into its glucoside, but on homogenization the ability to do so was reduced to a low level, but restored by the addition of UDPG. The fat body is the major site for this detoxication process, since its activity was shown to be about fifteen times that of the entire gut, and no activity was shown by Malpighian tubes, flight muscle or blood. Glucoside formation appears to be similar in vertebrates and insects as both employ UDPG, but reactions analogous to those in vertebrates involving UDP-acetl-

glucosamine or UDP-glucuronic acid could not be demonstrated by Smith and Turbert using locust fat body.

E. ASCORBIC ACID

Homogenates of fat body from the American cockroach, *Periplaneta americana*, can synthesize ascorbic acid using mannose, glucose, fructose, galactose or xylose as substrates (Rousell, 1956) and similar results have been obtained with the fat body from the cockroach *Leucophaea maderae*. The fat bodies from both of these species, however, contain intracellular symbionts, and the possibility existed that the observed ascorbic acid synthesis might be due to the microbial symbionts rather than to insect tissue enzymes. This was shown to be the case by some convincing experiments of Pierre (1962) in which the symbiont was isolated and grown on synthetic media, while cockroaches were obtained devoid of symbionts by feeding antibiotics. Homogenates of both the symbionts and of normal fat body from *Leucophaea maderae* were active in ascorbic acid synthesis, but negligible activity was present in the aposymbiotic fat body homogenate. There is no evidence at present that the cockroach requires ascorbic acid or derives any advantage from this biosynthetic ability of its symbionts.

IV. METABOLISM OF CARBOHYDRATES

A. THE GLYCOLYTIC PATHWAY

Recent reviews on the metabolism of carbohydrates in insects have been published by Chefurka (1959) and Gilmour (1961). It is now clear from the results of various workers that the Embden-Meyerhoff pathway of glycolysis is operative in insects; thus, Chefurka (1954) demonstrated the presence of ten essential enzymes of the pathway in a whole homogenate of adult houseflies (*Musca domestica*) and showed that all the phosphorylated intermediates of the pathway were metabolized by the homogenate. This whole-homogenate work gives no indication of the distribution of the enzymes, but some investigations have been done on individual tissues, muscle being most frequently used since it is a tissue in which a high glycolytic activity would be expected. No comprehensive and detailed study of glycolysis in fat body has been made on any one insect, although the occurrence of a number of the enzymes of the sequence have been demonstrated in the fat bodies of various species and there appears to be no reason to doubt that glycolysis occurs by the usual route in the fat body. The end products of anaerobic glycolysis may differ, however, from those in mammalian tissues and this point will be considered later.

1. *Evidence for the enzymes*

a. Phosphorylase. This was detected in the fat body of the silkworm by Shigematsu (1956) who followed the production of glucose-1-phosphate from glycogen and inorganic phosphate.

b. Phosphoglucomutase. This enzyme, which converts glucose-1-phosphate into glucose-6-phosphate was also found in silkworm fat body. At the equilibrium position, 87% of glucose-6-phosphate was found to be present. The corresponding figure for mammalian enzyme is 94·5%. Both enzymes are inhibited by fluoride.

c. Hexokinase. Shigematsu (1958b) has shown that glucose and fructose were converted into their 6-phosphates at relative rates of 1 : 1·46 by silkworm fat body preparations. The glucokinase activity was inhibited by fructose and the fructokinase activity partially inhibited by the simultaneous presence of glucose. The enzyme appears similar to the single non-specific hexokinase in locust muscle which was studied by Kerly and Leaback (1957). *Schistocerca* fat body will also readily convert glucose into phosphorylated derivatives in the presence of ATP (Candy and Kilby, 1961).

d. Glyceraldehyde-3-phosphate dehydrogenase and enolase. The presence of these have been shown in *Locusta* fat body by Delbrück *et al.* (1959) and the substrates shown to react at rates of 3000 and 70 μ moles substrate/h per g per fresh weight of tissue respectively.

There is little direct information about the occurrence in the fat body of the remaining enzymes of the glycolytic pathway but their existence can be inferred from the overall carbohydrate metabolism. The two enzymes below are of interest as they are associated with glycolysis in insects.

e. Glycerophosphate and lactate dehydrogenases. A most interesting feature of many insect tissues is the high α-glycerophosphate dehydrogenase (GDH) activity which is often found associated with a low lactic dehydrogenase (LDH) activity. Thus *Locusta* fat body was found to convert dihydroxyacetone phosphate into L-α-glycerophosphate at a rate of about 3000 μ moles/h per g fresh tissue, but pyruvate was reduced to lactate at only about 1% of this rate (Delbrück *et al.*, 1959). Two types of glycerophosphate dehydrogenase may be present in insect tissues as in the vertebrate; one is soluble and NAD-linked (GDH I) and the other is particulate and cytochrome-linked (GDH II).

Extracts of fat body of *Apis*, *Leucophaea*, *Melanoplus* and *Polyphemus* all show a high GDH I activity but no significant GDH II or lactic dehydrogenase activity, although exceptionally, the fat body of the blowfly *Phormia* contains GDH I, GDH II and LDH (Zebe, 1958). A

comparative study of enzyme activities in flight muscle, leg muscle and fat body of *Locusta* by Delbrück *et al.* (1959) showed that fat body resembled flight muscle in its GDH : LDH ratio, while leg muscle differed by having a LDH activity which was several times higher than the GDH one, and so much more closely resembled the pattern in vertebrate skeletal muscle. It is seen that care must be taken to avoid generalizing from one insect to others, or even from one tissue to others in the same insect.

2. *The end products*

The maintenance of glycolysis under conditions of oxygen insufficiency in vertebrate muscle is dependent upon the regeneration of the oxidized pyridine nucleotide from the reduced form which is produced at the triose phosphate dehydrogenation stage. This is normally achieved by coupling with the $NADH_2$-dependent reduction of pyruvate to lactate. In those insect tissues in which lactic dehydrogenase is virtually absent, the regeneration can be achieved instead by the reduction of dihydroxyacetone phosphate to α-glycerophosphate in the presence of GDH I (Fig. 2).

Vertebrate muscle

phosphoglyceraldehyde	NAD	lactate
phosphoglyceric acid	$NADH_2$	pyruvate

Overall reaction

$$\underset{\text{(glucose)}}{C_6H_{12}O_6} + 2ADP + 2H_3PO_4 = \underset{\text{(lactic acid)}}{CH_3CH(OH)COOH} + 2ATP + 2H_2O$$

Insect tissue

phosphoglyceraldehyde	NAD	α-glycerophosphate
phosphoglyceric acid	$NADH_2$	dihydroxyacetone phosphate

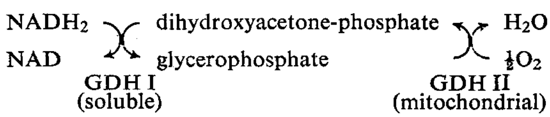
Overall reaction

$$\underset{\text{(glucose)}}{C_6H_{12}O_6} + H_3PO_4 = \underset{\text{(pyruvic acid)}}{CH_3COCOOH} + \underset{\text{(glycerophosphate)}}{CH_2OH.CHOH.CH_2OPO_3H_2} + H_2O$$

FIG. 2. End products of glycolysis.

Chefurka (1958b, 1959) found the activities of lactate and α-glycerophosphate dehydrogenases in cockroach coxal muscle to be 10 and 3206 units/g wet weight/min respectively, and when fructose-1-6-diphosphate was incubated anaerobically with coxal muscle, 1 mole of pyruvate and 1 mole of glycerophosphate were produced for each mole of fructose diphosphate utilized. Negligible amounts of lactate were formed, thus confirming the overall scheme (Fig. 2). The glycerophosphate was isolated and identified by paper chromatography. As cockroach fat body was

found to have a similar preponderance of GDH I over LDH (activities 292 and 20 units respectively), it can be anticipated (in the absence of direct experiment) that fat body tissue would show a similar result. It will be noted, however, that the overall process in such insect tissues produces one molecule of ATP per glucosyl unit in glycogen metabolized and is thus less efficient for the production of energy than the vertebrate scheme, where each glucosyl unit gives rise to three molecules of ATP. If trehalose or glucose is the starting material and is converted into glycerophosphate and pyruvate, there can be no net production of ATP because one molecule is required for the phosphorylation of the glucose to start the process. This is presumably of little importance, since conditions generally in insect tissues will be aerobic, and this pathway of glycolysis will serve to supply three carbon compounds for oxidative metabolism. The fate of the glycerophosphate, at least in flight muscle, is a rapid reoxidation back to dihydroxyacetone phosphate by the active mitochondrial enzyme (GDH II), a reaction in which the equilibrium favours dihydroxyacetone phosphate, and which is coupled to phosphorylation (P/O found by Sacktor and Cochran, 1958, to be 1·0–1·7). Thus a cycle is postulated in flight muscle:



The overall reaction serves for the oxidation of $NADH_2$, and the three-carbon compounds are acting catalytically; the ultimate oxidation of carbohydrate to carbon dioxide and water with concomitant energy release proceeds through pyruvate and the Krebs cycle. Chance and Sacktor (1958) suggest that the Krebs cycle may not carry a significant part of the flight muscle metabolism since intermediates of the cycle were metabolized at a very much slower rate than glycerophosphate by mitochondrial preparations (e.g. the respiratory rate with pyruvate was only 2% of that with glycerophosphate as substrate). Gregg *et al.* (1960) dispute that insect mitochondria have these unique properties and consider that the results were a consequence of the mode of isolation of the mitochondria, since their own preparations of insect mitochondria metabolized pyruvate, Krebs cycle compounds and glycerophosphate at similar rates and with high P/O ratios.

The frequency of the wing beat in insects is often very high—about 14 000 cycles a minute in *Drosophila*—and the associated muscular activity may raise oxygen consumption up to one hundred times the resting level; in *Lucilia*, for example, the rate of oxygen consumption during

flight may reach 3000 μl/g/min (Davis and Fraenkel, 1940). Since the energy for flight is derived almost exclusively from carbohydrate utilization, the muscles must possess enzyme systems which are capable of high activity when required, but which operate during rest at only a few per-cent of their maximum. Fat body tissue is not required to produce large amounts of chemical energy for work, but, as mentioned earlier, it has to synthesize trehalose at about the same rate as the muscles are utilizing it. Much of the detailed work on carbohydrate metabolism in insects has been done using either homogenates of muscle or preparations of flight muscle sarcosomes, and it would appear unwise to assume that similar findings would be true for fat body tissue or fat body mitochondria in view of the different aspects of carbohydrate metabolism expected in the two differing tissues.

B. THE PENTOSE PHOSPHATE PATHWAY

The operation of this cycle has been demonstrated in various insects, for example, in the pea aphid (Newburgh and Cheldelin, 1955) in *Phormia* (McGinnis *et al.* 1956), in the honey bee (Hoskins *et al.*, 1956) and in considerable detail in the common house fly, by Chefurka (1957, 1958a). In all these cases, however, homogenates of whole insects were used although Chefurka (1955) has also shown that the cycle is operative in homogenates of fly thoraces, which may be regarded as a crude thoracic muscle preparation. No very detailed examination of fat body tissue for pentose cycle enzymes has been reported, although glucose-6-phosphate dehydrogenase has been found in the fat body of *Schistocerca* (Fenwick, 1958a) and of *Blattella* (Young, 1958a), and 6-phosphogluconic dehydrogenase was also found in *Blattella*. These enzymes are NADP linked. Young observed that the oxygen uptake of whole homogenates of fat body was only very slightly raised above the endogenous rate on addition of glucose-6-phosphate or fructose 1-6-diphosphate, suggesting that the pentose cycle plays only a minor role in fat body metabolism.

C. POLYOL DEHYDROGENASE

A dehydrogenase linked to NADP has been found by Faulkner (1958) in the fat body, blood and other tissues of the silkworm, which reduces a number of hydroxyaldehydes and carbonyl compounds to the corresponding alcohols. Among the substrates which are reduced *in vitro* are glycolaldehyde, glyceraldehyde, D-erythrose, glucuronic acid, galacturonic acid, ribose-5-phosphate and glyoxal, but hexoses and pentoses are not attacked. The function and natural substrate of the enzyme are

not known, but Faulkner has suggested that the enzyme may well be important in the formation of glycerol from glyceraldehyde, since glycerol is a compound found in the blood of some insects, sometimes in high concentrations, as in a parasitic larva, *Bracon cephi*, investigated by Salt (1958) where the concentration reaches 20%.

V. Tissue Respiration

A. The Tricarboxylic Acid Cycle

This cycle is now recognized as the major terminal metabolic pathway for biological oxidation in an extremely wide range of animal, plant and bacterial cells, and during the last decade a considerable amount of work has been done in order to see if it is also operative in insect tissues. Levenbook (1961) has shown the presence of all the enzymes of the cycle (Fig. 3)

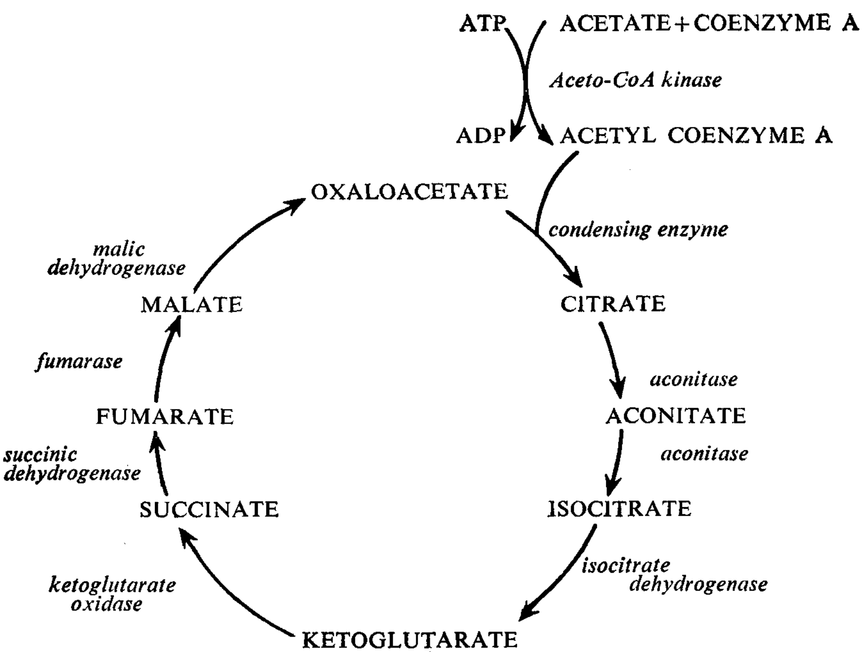


Fig. 3. The tricarboxylic acid cycle.

in acetone powder extracts, mitochondrial preparations and homogenates prepared from whole *Prodenia* larvae. The only two insect tissues which have been examined in any detail are muscle and fat body, and the former

has more often been the tissue of choice for experimental work as it shows high metabolic activity, and the working of the cycle appears to be well established in this tissue. All the necessary enzymes for the cycle have not yet been detected in the fat body derived from any single insect species, but if the findings of various workers on different fat bodies are considered together, there appears little doubt that the cycle operates in fat body also. Some of the evidence for the individual enzymes is as follows.

a. Aceto-CoA kinase. Levenbook (1961) demonstrated that fat body from *Prodenia* could activate acetate by trapping the product with hydroxylamine and establishing the identity of the acetohydroxamate by chromatography.

b. Condensing enzyme. This was shown to be present in *Locusta* fat body by Zebe (1959), but the activity per g tissue was very low and only about 2% of that in flight muscle. Hearfield and Kilby (1958) were unable to detect it in *Schistocerca* fat body. Clements (1959), however, demonstrated the production of $^{14}CO_2$ from sodium acetate-2-^{14}C in this tissue and the process was 95% inhibited by fluoroacetate. This compound is known to function by a metabolic conversion to fluorocitrate (a process involving aceto-CoA kinase and the condensing enzyme), which inhibits aconitase and so prevents the smooth oxidation of acetate by the cycle. The production of the inhibition thus implies the intervention of the condensing enzyme. The accumulation of citrate has been demonstrated directly by Levenbook (1961) when whole *Prodenia* larval preparations were incubated with acetyl-CoA and oxalacetate, but no experiments on individual tissues were reported. The condensing enzyme is very active in locust flight muscle, the amount per mg nitrogen being about 130 times that in rat leg muscle (Zebe, 1960).

c. Aconitase. Present in fat bodies from *Prodenia* and *Schistocerca*, Levenbook (1961) observed that isocitrate was dehydrated three times as fast as citrate, indicating that the position of equilibrium is well over to the citrate side, as with the mammalian enzyme.

d. Isocitric dehydrogenase. This NADP-dependent dehydrogenase was found in *Schistocerca* fat body by Hearfield and Kilby (1958) and also by Fenwick (1958b), who showed that it was present in the supernatent fraction after spinning a homogenate at 14 000 *g* and completely absent from the particulate fractions. On the other hand, Young (1959) found it in the "pellet" fraction of *Periplaneta* fat body homogenate which had been spun at 8500 *g*.

e. α-Ketoglutarate oxidase. Increases in oxygen uptake by fat body homogenates on addition of α-ketoglutarate have been observed by Bellamy (1958) using *Schistocerca* (Table I), and by Young (1959) with *Periplaneta*,

Blattella, *Acheta*, *Galleria* and *Tenebrio*. The fat body enzyme has not been examined in detail but is probably similar to that in a mitochondrial preparation from the honey bee, which requires NAD, Co-enzyme A, magnesium ions and thiamine pyrophosphate (Hoskins *et al.*, 1956).

f. Succinic oxidase complex. Sacktor and Thomas (1955) found a succinate-cytochrome *c* reductase in all tissues of the American cockroach which they examined by using the method in which the reduction of cytochrome *c* is followed in the presence of cyanide (to inhibit cytochrome oxidase). The activity in the fat body was in the middle range, being similar to that in midgut and Malpighian tubes but less than that in muscle or hindgut. By following the increase in oxygen uptake on addition of succinate to an homogenate, the succinic oxidase complex has been shown to be present in the fat body of *Bombyx* pupae (Shigematsu, 1956), *Schistocerca* (Bellamy, 1958) and *Periplaneta* (Young, 1959).

TABLE I

Respiratory rates for *Schistocerca* fat body (data from Bellamy, 1958).

System	Q_{O_2}
Intact fat body tissue	7·8
Homogenized fat body	1·7
,, ,, ,, + succinate	4·1
,, ,, ,, + α-ketoglutarate	2·4

It will be seen that the endogenous respiration of the tissue is reduced to about a quarter of its value by the process of homogenization, and the addition of substrates will only raise it to about half its original value, suggesting that the enzyme is labile on homogenization. It appears unlikely that the reduced respiration was due to a dilution effect on coenzymes, since the Q_{O_2} of other tissues (e.g. leg, thoracic muscle) was actually slightly raised after homogenization.

Clements (1959) has observed that the production of $^{14}CO_2$ from labelled succinate by *Schistocerca* fat body was reduced by about 95% on homogenization, and considered that this might be due to the release of some inhibitor. It is known that the succinic oxidase activity of rat heart muscle is reduced by the addition of a homogenate of rat gastric intestinal mucosa, due to the presence in the latter of inhibitory material which appears to be free unsaturated fatty acid in type. (Nakamura *et al.*, 1959). Clements could not observe any inhibition, however, of sheep heart succinoxidase by a locust fat body homogenate. George and Hegdekar (1961) found that when sheets of whole fat body from *Schistocerca* (and

three grasshoppers) were treated with neotetrazolium salt (an indicator dye for the histochemical demonstration of succinic dehydrogenase activity), positive results were obtained because deep purple diformazan granules appeared in the cells, being distributed at the periphery and around the fat globules. Sections from frozen fat body or air-dried whole fat body were inactive, as indeed was tissue treated with fat solvents such as cold ether, acetone and ethyl acetate, again showing how easily the activity of the enzyme can be lost by experimental manipulation. A similar technique had previously been used to demonstrate the presence of succinic dehydrogenase in the isolated mitochondria of *Periplaneta* fat body (Pearse and Scarpelli, 1958) but a positive result could not be obtained by a similar preparation from *Locusta* fat body (Pearse, 1959).

g. Fumarase. Its presence was shown in *Schistocerca* fat body by Hearfield and Kilby (1958).

h. Malic dehydrogenase. This occurs in the fat body of *Schistocerca, Blattella* (Young, 1958a), *Periplaneta* (Gary *et al.*, 1959) and *Locusta* (Delbrück *et al.*, 1959). The enzyme is NAD-linked. It shows a two-and-a-half fold variation in activity during the moulting cycle of *Periplaneta,* reaching a maximum at the end of the first quarter.

The formation of labelled glutamate, aspartate and alanine by the incubation of locust fat body with acetate-2-^{14}C (Clements, 1959) is good circumstantial evidence for the operation of the cycle, since these three amino acids would be expected to appear if acetate entered the cycle and gave rise to labelled α-ketoglutarate, oxalacetate and pyruvate and these then underwent transamination.

The blood citrate concentration in insects has been examined by Levenbook and Hollis (1961) and found to be so much higher than that in other organisms, that they consider it a "biochemical characteristic" of the class Insecta. The high level is observed even in insects whose diet is very low in citrate (e.g., dipterous larvae feeding on meat) and there seems little doubt that the citrate is largely endogenous in origin and is formed and metabolized by the operation of the tricarboxylic acid cycle. Information is lacking at present about the site of its formation in the insect body, and it would be interesting to know if the fat body, which synthesizes blood trehalose and blood globulin, also supplies the blood citrate.

B. TERMINAL RESPIRATORY PATHWAY

A detailed study of the cytochromes present in different tissues of the silkmoth, *Platysamia cecropia*, has been made by Shappirio and Williams (1957a) using the technique of low temperature spectroscopy. The pattern of the cytochrome system of fat body and somatic muscle showed

interesting differences. Cytochromes $a+a_3$, b and c were present in both tissues, but larval and adult fat body in addition contained relatively high concentrations of cytochrome b_5 which was undetectable at all stages in muscle. Another striking difference was that while the pattern and relative concentrations of the cytochromes in muscle remained unchanged during development, the pattern in pupal fat body differed from that in larval and adult tissue by the complete disappearance of cytochromes b and c, and by the relatively low concentrations of cytochromes $a+a_3$ and b_5.

Cytochrome b_5 was discovered by Williams in 1947 working with insect tissue, but is now known to be widely distributed in non-muscular tissue in invertebrates and vertebrates. This cytochrome has been shown to be associated with the microsomal fraction in contrast to the "classical" cytochromes $a+a_3$, b and c which occur in mitochondria. In the Cecropia silkworm, there is a pupal diapause about 8 months long, which is initiated by the insect's endocrine system becoming inactive. The decline in concentration of the cytochromes in the fat body is thought by Shappirio and Williams to be due at least to a partial destruction of the endoplasmic reticulum and mitochondria at the beginning of diapause. These organelles can still be isolated from diapausing non-muscular tissue but in small amounts only, and the mitochondria then show only bands corresponding to cytochromes $a+a_3$ and the microsomes only cytochrome b_5. Diapause is terminated by the prothoracic gland becoming reactive and liberating the hormone ecdyson which stimulates the resynthesis of cytochromes b and c, and an increase in concentration of cytochromes $a+a_3$ and b_5, so that the larval pattern reappears in the adult tissue. The very low respiratory rate of tissues during diapause, and their remarkable insensitivity to poisons such as cyanide and carbon monoxide (in contrast to their sensitivity during the larval and adult stages) have been discussed by Shappirio and Williams (1957b) in terms of the differing cytochrome patterns.

Three enzymes associated with the terminal respiratory chain have been found in fat body tissue. The succinic-cytochrome c reductase has already been mentioned. The presence of a NAD-cytochrome c reductase has been shown in the fat body of *Schistocerca* (Kilby and Neville, 1957) and in *Calliphora* (Desai and Kilby, 1958a).

The cytochrome oxidase activity of *Periplaneta* tissues has been examined by Sacktor and Bodenstein (1952) and that of fat body was found to be relatively low compared with most other tissues. This small numerical value is in part due to the activity being expressed per mg dry weight or per mg nitrogen, values which are high due to the presence of fat and

urates. Sacktor and Bodenstein commented that the cytochrome activity of various tissues shows a close correlation with the abundance of tracheation as observed by Day (1951); the fat body has the least tracheation and one of the lowest cytochrome oxidase activities of all tissues. The cytochrome oxidase activity of fat body tissue is not, however, constant during development and in the fifth stadium of *Schistocerca*, for instance, it was found by Hearfield and Kilby (1958) to be relatively high at the beginning of the stadium and falling to one-thirtieth of its value towards the end, when it rose suddenly at the time of the fifth and final moult. The decrease of the cytochrome oxidase band (a_3) during the pupal diapause of *Platysamia* was mentioned above.

C. THE GLYOXALATE CYCLE

This cycle makes possible the formation of succinate from acetate, i.e. a C_4 compound from two C_2 units. It operates in bacteria and higher plants but was considered not to occur in animal tissues, as one of the key enzymes, isocitric lyase (which splits isocitrate into succinate and glyoxalate), had not been detected. Levenbook (1961) was unable to demonstrate it in homogenates of whole mature larvae of *Prodenia*, but Carpenter and Jaworski (1962) pointed out that the enzyme had not been found previously in tissues known to be synthesizing fat, but rather in ones which are utilizing fat or acetate. Sixth instar larvae are probably synthesizing fat and they suggested that prepupae or pupae, which are utilizing fat, would be a more suitable stage to study. Activity was detected at these stages although it was very low when compared with, say, that in the castor-bean endosperm. They confirmed that activity was lacking in sixth instar larvae and found that it appeared in the prepupae, reached a maximum in 1-day-old pupae, and declined to zero by the time the pupae was 5 days old. This represents the first report of the occurrence of isocitric lyase in insects, and indeed in the animal kingdom. It would be interesting to know if the enzyme occurred at any time in the fat body, a site of fat metabolism, and whether the other characteristic enzyme of the glyoxalate cycle, malate synthetase (which forms malate from acetate and glyoxalate) is also present and shows a similar variation in activity during development.

Experiments made by Bade (1962) to try and detect malate synthetase in pupating cecropia silkworms, however, led to the conclusion that the enzyme was not active in the fat body and that the citric acid cycle operated as the major pathway for acetate metabolism, and that the glyoxalate cycle enzymes had no appreciable activity. There have been repeated assertions in the literature that insects can convert fat into carbohydrate, but

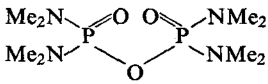
as Bade and Wyatt (1962) have recently pointed out, the analyses on which these conclusions were based were inconclusive, because the presence of trehalose was not considered; incorrect assumptions were made about the nature of reducing material in the blood and so on. They were able to show that the apparent increase of glycogen and free sugars at the time of the pupal moult could be accounted for by the conversion of material from the cuticle, and it is no longer necessary to postulate the conversion of lipid into carbohydrate. Had this been necessary, some mechanism for the incorporation of acetyl CoA from the metabolism of fats into carbohydrates would be required, such as the operation of the glyoxalate cycle.

D. OTHER ENZYMES

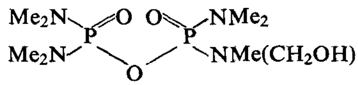
1. *Anticholinesterase activators*

The organophosphorus insecticide schradan (II) has a very low anticholinesterase activity *in vitro*, but can be converted by mammalian and insect tissues into a powerful inhibitor of cholinesterase and other esterases. This activation has been demonstrated by incubation of schradan with liver slices or insect fat body. A study of the enzyme system responsible in *Schistocerca* fat body was made by Fenwick (1958a), who was able to obtain an active cell-free extract. Oxygen was required for the activation, and both the particulate and the soluble fraction of the extract were necessary. The soluble fraction was shown to contain at least three enzymes (glucose-6-phosphate dehydrogenase, isocitric dehydrogenase and the malic enzyme) which could form reduced NADP through oxidation of endogenous substrates, and if reduced NADP was added to the activating system, the soluble fraction was then no longer necessary. The requirement of the schradan-activating enzyme for both oxygen and the *reduced* form of the coenzyme indicates that it is a mixed function oxidase, in which one atom of molecular oxygen is reduced to water (in the present case by $NADPH_2$) and the other atom is incorporated into the substrate. It is probable that schradan is converted by the activating enzyme into a methylol derivative (III) (Spencer and O'Brien, 1957). The same enzyme will activate dimefox, (*bis*dimethylamino fluorophosphine oxide, $(NMe_2)_2POF$), a similar chemical modification probably being produced. Fenwick has discussed possible mechanisms for the simultaneous oxidation of schradan and the reduced coenzyme, by schemes involving metal ions at the active centre of the enzyme, but as it has not yet been possible to solubilize and purify the enzyme, an absolute requirement for a metal cannot be demonstrated. Inhibition of the system by agents such as mepacrine and chelating compounds suggest that a

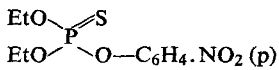
flavine prosthetic group and a metal (? iron) may be involved. Separation of the constituents of the fat body cell by differential centrifugation has shown that the enzyme system is largely located on the microsomes (84 %), and the rest is on the mitochondria, while the soluble fraction was without activity (Fenwick, 1958b).



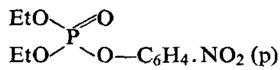
II. Schradan



III. Activated Schradan



IV. Parathion



V. Paraoxon

The insecticide parathion (O, O-diethyl-O-nitrophenyl thiophosphate, IV) is another example of a compound which, when pure, does not possess anticholinesterase activity *in vitro*, but which can be converted into a powerful inhibitor by insect tissues, both *in vivo* and *in vitro*. Kok and Walop (1954) studied the effect of incubation of various tissues of *Periplaneta* with parathion, and found that fat body showed the highest activity in the conversion into an anticholinesterase, which is believed to be paraoxon (V). It is interesting to note that mammalian tissues can bring about a similar activation of organophosphorus compounds and that the liver is the most active tissue as in the activation of schradan (Gardiner and Kilby, 1952) and parathion (Diggle and Gage, 1951), thus providing another point of resemblance between the insect fat body and the mammalian liver.

2. *The malic enzyme*

This enzyme is well known from sources such as pigeon liver and wheat germ, and was first found in insects by Faulkner (1956) where it is active in the blood. It catalyses the reaction

$$\text{malate}^{--} + \text{NADP}^{+} = \text{pyruvate}^{-} + CO_2 + \text{NADPH}$$

and is considered by Faulkner to help to maintain the ionic balance and redox potential of the blood. It has been found in the fat body of *Schistocerca* by Fenwick (1958a).

VI. Lipids and the Fat Body

A. Composition of Insect Lipids

Numerous analyses of the composition of insect fat have been published, but almost all are for total body fat and not for that extracted from

isolated fat body tissue. An exception is a study by Fawzi *et al.* (1961) who prepared samples of total body fat by solvent extraction of whole locusts, and also of stored fat by homogenizing and centrifuging fat body tissue and extracting the fat layer with solvent. Analyses showed small differences in the compositions of the two samples; for instance, the stored fat contained slightly more arachidonic acid and slightly less linoleic acid than the total body fat, while the latter contained phospholipids which were absent from fat body lipid. However, as the major part of the fat reserves are found in the fat body, analyses of whole body fat can be taken without serious error as indicative of the composition of fat in the fat body itself. The amount of fat present in insects is often surprisingly high, making up, for example, 25 % of the wet weight of the cocoon of the silkmoth (and forming the "chrysalis oil" of commerce), and 49–69 % of the dry weight of male locusts (Fawzi *et al.*, 1961). The larvae of the beetle *Pachymerus dactris*, which feed on the endosperm of palm nuts, is reported to contain nearly 50 % of fat (Collin, 1933). Reviews on insect fat have been made by Gilmour (1961) and Hilditch (1956).

It is difficult to generalize about the lipid composition in so diverse a group as the insects, as the published information is fragmentary and exceptions are not uncommon. While the lipid is generally composed of neutral glycerides, free fatty acids may make up 75 % of the lipid fraction (as in *Melanopus atlantis*, a Mexican Orthopteran) (Giral *et al.*, 1946) or even more, as in another Mexican species, *Sphernarium purpurascens* (Giral, 1946). These observations were made on insects which had been killed with petroleum ether and then dried in the sun, and in view of the high lipase activity in the fat body (George and Eapen, 1959b), it might be desirable to check this high content of fatty acid by analyses made on lipid extracted immediately after death. Many insect fats contain large amounts (60–85 %) of unsaturated fatty acids, largely palmoleic, oleic, linoleic and linolenic acids, while palmitic and stearic acids represent the main long chain saturated acids (15–35 %). Shorter chain acids are usually absent; but exceptionally, an aphid *Pemphigus* sp. has a fat composed almost entirely of saturated fatty acids, including short chain ones like butyric and caprylic (Timon-David, 1928). The oil from a Mexican orthopteran, *Taeniopoda auricornis*, is poisonous to rats and it has been suggested that this is due to the presence of sulphur compounds, which may be neutral sulphuric esters of high molecular weight alcohols (Giral *et al.*, 1946).

The composition of the fat in insects may sometimes resemble that of the diet, as in the midge, *Tanytarsus lewisi*, where the fat of adults contains polyenoic acids similar to those in the algae and plankton on which

the midge larvae had lived (Grindley, 1952). But, in contrast, the larvae of *Pachymerus* lay down a fat which contains nearly four times as much unsaturated fatty acid and only one-half as much lauric acid as are present in the fat of the palm nuts on which they feed. There may thus be considerable metabolic modification of dietary fat before storage and synthesis *de novo* from non-fatty components in the diet. An example of change in the nature of the fat during growth is afforded by the sugar beet webworm, *Loxostege sticticalis,* in which the amount of linolenic acid falls from 26% at the end of the fifth instar to 0·8% in the fertile female and to zero in the sterile female (Pepper and Hastings, 1943).

The development of vapour phase chromatography has made the analysis of complex mixtures of fatty acids much easier and quicker than previously, and the time may be opportune for a fairly systematic survey of the nature of the fatty acids over the whole insect kingdom. It would be of great interest to see if there are quantitative and qualitative patterns in the fats from orders or families, or whether the nature and quantity of the fats are determined by their dietary source. One might ask if the anomalous fatty acid content of *Pemphigus* is characteristic only of the few particular species examined, or of the genus, or of Aphididae in general, or whether it is found elsewhere in *Hemiptera.*

There is very little information about other types of lipids which might be expected to occur in the fat body, as the analytical studies on insects that have been published have usually used whole insects as a starting material. One of the most comprehensive of such studies is that of Wren and Mitchell (1959) who separated and identified a large number of lipid components from *Drosophila melanogaster.*

Coupland (1957) has shown by staining that there is a small amount of phospholipid in the fat body cells of *Schistocerca gregaria,* although very much more is present in oenocytes.

B. FATTY ACID BIOSYNTHESIS

1. *Acetate incorporation*

Evidence was obtained by Zebe and McShan (1959a) for the existence in fat body of an active enzyme system for the biosynthesis of fatty acids. Intact fat body from *Prodenia* was carefully removed to avoid damage and was shown to incorporate labelled acetate readily into long chain fatty acids. Homogenization decreased the activity to a third or a quarter of its original value, and only a partial restoration was possible by the addition of various cofactors. The effect on the efficiency of acetate incorporation of leaving out each one in turn is shown in Table II.

It was reported that manganous ions, isocitrate and glucose-1-phosphate were not required, and if the glutathione in the complete system was replaced by cysteine, the activity increased by about 50%. Similar results were obtained by Tietz (1961) using fat body from the migratory locust, *Locusta migratoria* (Table II). There is agreement that ATP, coenzyme A, malonate and an -SH compound are required, and that NAD and Mn^{++} are without effect. Magnesium ions are essential with *Locusta* homogenate, and presumably also with the *Prodenia* one, but this was not revealed by Zebe and McShan's experiments as they prepared their homogenates in a buffer solution containing magnesium chloride. The *Locusta*

TABLE II

Cofactor requirement for acetate incorporation by *Prodenia* and *Locusta* fat bodies

Cofactor omitted	Acetate incorporated into fatty acids. Complete system 100	
	Prodenia	*Locusta*
None	100*	100†
ATP	25	15
CoA	20	42
glutathione	30	51
malonate	35	3
NAD	85	100
NADP		57
$MgCl_2$	—	3
$MnSO_4$	100	96
α-ketoglutarate	100	60
$KHCO_3$	—	24

* Complete system contained 1-^{14}C acetate, ATP, CoA, glutathione, malonate, NAD, NADP, and homogenate in a buffer containing KCl and $MgCl_2$ pH 7·2 (data from Zebe and McShan, 1959a).

† Complete system contained 1-^{14}C acetate, ATP, CoA, glutathione, malonate, NAD, ADP, $MgCl_2$, $MnSO_4$, $KHCO_3$ and homogenate, pH 7·0 (data from Tietz, 1961).

preparation appears to be the more sensitive in revealing cofactor requirements and shows that NADP, α-ketoglutarate and carbon dioxide are also required. These requirements are very similar to those needed to fortify liver extracts (Porter *et al.*, 1957) and mammary gland extract (Popjak and Tietz, 1954), so a similar pathway of acetate incorporation may be operative in the various systems. The scheme proposed by Wakil and Ganguly (1959) for fatty acid synthesis is shown in Fig. 4. This

accounts for most of the cofactor requirements observed. The activation of acetate requires ATP and Co-enzyme A, while the formation of malonyl-CoA needs carbon dioxide which is released again in stage (7). Ketoglutarate, or some other member of the tricarboxylic acid cycle, may be required to reduce the added NADP to give the reduced nucleotide necessary for stages (4) and (6). The requirement for malonate is unexplained.

$$\mathrm{CH_3COOH} \xrightarrow[(1)]{\mathrm{CoA{-}SH + ATP}} \mathrm{CH_3CO{-}S.CoA} \xrightarrow[(2)]{\mathrm{CO_2 + ATP}} \underset{\displaystyle \mathrm{COOH}}{\mathrm{CH_2CO{-}S.CoA}}$$

$$\xrightarrow[(3)]{\mathrm{CH_3CO{-}S.CoA}} \mathrm{CH_3CO.}\underset{\displaystyle \mathrm{COOH}}{\mathrm{CH}}\mathrm{.CO{-}S.CoA} \xrightarrow[(4)]{\mathrm{NADPH_2}} \mathrm{CH_3CH(OH).}\underset{\displaystyle \mathrm{COOH}}{\mathrm{CH}}\mathrm{.CO{-}S.CoA}$$

$$\xrightarrow[(5)]{-\mathrm{H_2O}} \mathrm{CH_3CH{:}}\underset{\displaystyle \mathrm{COOH}}{\mathrm{C}}\mathrm{.CO{-}S.CoA} \xrightarrow[(6)]{\mathrm{NADPH_2}} \mathrm{CH_3CH_2.}\underset{\displaystyle \mathrm{COOH}}{\mathrm{CH}}\mathrm{.CO.S.CoA}$$

$$\xrightarrow[(7)]{-\mathrm{CO_2}} \mathrm{CH_3CH_2CH_2CO{-}S.CoA} \xrightarrow[\text{as stage (3) etc.}]{\text{reacts with malonyl CoA}}$$

FIG. 4. Scheme proposed for fatty acid biosynthesis (Wakil and Ganguly, 1959).

Tietz examined the distribution of the enzyme system in his *Locusta* fat body homogenate and found that under optimal conditions a particle-free supernatent was as active as the whole homogenate in incorporating acetate into fatty acids, although Zebe and McShan found activity about equally divided between supernatant and sediment in their homogenate from *Prodenia*. The acetate-activating enzyme was shown by Tietz (by acethydroxamic acid formation) to occur in the supernatant, while the particles were inactive. On the other hand, malonate was not activated by supernatant, but the particles were active and malonomonohydroxamic acid was identified. Using labelled carbon dioxide, it was shown that both supernatant and particles were necessary for fixation of carbon dioxide (91 % of the label being found in malonate), while each fraction alone was inactive. It is difficult at present to account for the requirement for malonate and carbon dioxide by the supernatant as it appears that the supernatant is neither able to activate malonate nor to fix carbon dioxide.

In experiments with *Locusta* homogenates, acetate was shown to be incorporated only into fatty acids present as glycerides (81 %) or phospholipids (18 %) and negligible amounts occurred in the unsaponifiable material (e.g., sterols) or as free fatty acids. With both *Prodenia* and *Locusta*, the major part of the labelled acetate was incorporated into palmitic acid, and smaller amounts only into short chain acids (Table III).

It is interesting that long chain saturated fatty acids should predominate, since it is known that *in vivo*, 65–70% of the acids are unsaturated ones.

TABLE III

Incorporation of labelled acetate into fatty acids by *Prodenia* and *Locusta* fat bodies

Fatty acid	Acetate incorporated (%) *Prodenia*	*Locusta*
Stearic (C_{18})	8	22
Palmitic (C_{16})	78	60
Myristic (C_{14})	3	5
Lauric (C_{12})	1	3·5
Shorter + unsaturated	9	11

2. *Fatty acid synthesis from non-lipid nutrients*

It has been known for a long time that fat could be formed by insects from a wide variety of foodstuffs. This was clearly illustrated by the experiments of Wigglesworth (1942) in which *Aëdes* larvae were starved until the fat reserves in the fat body were exhausted, and the reappearance of fat droplets was observed after feeding with protein, amino acids and sugars. The fat was mainly located in the cells of the fat body (although some was laid down in the oenocytes), and it appeared that the fat was being synthesized *in situ* in these cells in the neighbourhood of mitochondria. Direct evidence that the fat body *per se* is able to synthesize fat from various substrates was given by Clements (1959) who studied the incorporation of various labelled materials when incubated with intact fat body tissue from *Schistocerca*. Glycine-^{14}C (G) was partially oxidized on incubation (70% of the label appearing as carbon dioxide) and partially incorporated, and of the latter, about one-quarter was in the lipid fraction where it was present almost entirely in neutral fat. Hydrolysis of this showed that just over half of the label was in the fatty acid portion of the molecule. Leucine-^{14}C (G) gave similar results but with somewhat lower incorporation into fat. Glucose-^{14}C (G) was incorporated to the extent of 70%, about half of this being into trehalose and about a quarter into the lipid fraction. Zebe and McShan (1959a) also found that generally labelled glucose gave rise to labelled fatty acids (using *Prodenia* fat body) but the amount of fatty acids formed was not as large as when labelled acetate was used. The cofactors required with glucose appeared to be the same as for acetate incorporation. At present the indications are that the fat body can utilize carbohydrate, amino acids or acetate for the

synthesis of fatty acids, but there are points which require further elucidation, such as the role of malonate and the origin of the unsaturated acids. The fat body is not, however, unique in its ability to synthesize fats, as in addition to the appearance of fat in oenocytes mentioned above, flight muscle from *Prodenia* was also observed by Zebe and McShan to be able to incorporate acetate into fatty acids.

Van Handel and Lum (1961) have made the interesting observation that while the female mosquito can synthesize large amounts of triglycerides when fed on glucose, male mosquitoes and both sexes of house flies are unable to do so. Although both male and female mosquitoes contained similar amounts of fat at emergence (around 60 μg per insect), after feeding on glucose for 7 days, the females contained about 700 μg but the males only 10–20 μg. Thus a fifty-fold difference in the triglyceride content of the sexes has developed, although both had fed freely on the glucose provided. Further investigation, the authors suggest, might facilitate the study of the physiological factors that control lipogenesis *in vivo*. The nature of the fatty acids synthesized from glucose was examined and it was found that 92% was accounted for by palmitic, palmitoleic and oleic (approximately 3:3:2 by wt.). Although the experiment extended over 10 days, no significant amounts of polyunsaturated acids were formed from glucose, yet linoleic acid is a normal component in mosquito fat; possibly it is derived from the natural diet and the mosquito is unable to synthesize it. The very low incorporation of acetate into unsaturated acids observed in the experiments with *Prodenia* and *Locusta* mentioned earlier may have been due to the short duration of the experiment—analyses being made after 2 h incubation of the fat body with acetate *in vitro*, and desaturation may be a slower process which was not observed under the experimental conditions used.

3. *Breakdown of fat*

The lipid in the fat body is an energy reserve which can be mobilized rapidly during starvation or to sustain continuous muscular activity, as in *Schistocerca*, which derives two-thirds of the energy expended during prolonged flight from fat metabolism (Weis-Fogh, 1952). The presence of lipase in fat body would thus be expected, and was demonstrated histochemically in *Rhodnius* fat body by Wigglesworth (1958) and in *Schistocerca* fat body by George and Eapen (1959a).

The distribution of lipase in the fat body of *Rhodnius* was studied histochemically by Wigglesworth who used 5-bromoindoxyl acetate with copper sulphate as a substrate which readily penetrates into cells and which on hydrolysis gives a blue deposit of indigoid dye at the site of

location of the lipase. He found that each fat droplet, irrespective of size, had its own little point or cap which was the site of reaction, and there were also some exceedingly fine filaments which stained blue when applied to the surface of the nuclei, as well as a few filaments in the cytoplasm. The enzyme was not inhibited by anticholinesterases such as 10^{-4}M eserine and only partially by 10^{-4}M paraoxon and thus appeared to be a true lipase, similar to that in the mammalian pancreas, rather than a cholinesterase. Wigglesworth suggested that the sharply localized caps of lipase on the fat droplets, catalysing the reversible hydrolysis of triglycerides, control the transfer of fat to and from the storage droplets. He considered the significance of the filaments to be much more doubtful; the evidence is against their being artifacts, and they might possibly represent sites of intracellular cathepsins, since these enzymes will also hydrolyse 5-bromoindoxyl acetate. Alternatively, they might be involved in phospholipid metabolism.

There is some experimental evidence for the existence in the fat body of at least two of the five enzymes necessary for the degradation of fatty acid to acetyl coenzyme A. Young (1959) showed that *Periplaneta* fat body homogenate gave a small increase in oxygen uptake on incubation with β-hydroxybutyrate and that this was raised slightly by addition of NAD and coenzyme A, suggesting the presence of a β-hydroxyacyl dehydrogenase. Zebe (1959) found β-ketoacyl thiolase in *Locusta* fat body.

Quantitative assay of lipase by manometry with tributyrin as substrate has shown that the activity of *Schistocerca* fat body is high—more than twice that of pigeon adipose tissue and much higher than that of *Schistocerca* flight muscle (George and Eapen, 1959b). The fat body showed the highest specific activity (units per g wet weight) for glycerokinase of any of the seven tissues from *Locusta* which were examined by Zebe, and it is interesting to note that the enzyme appeared to be completely absent from flight muscle. On the other hand, the specific activities of β-ketoacyl thiolase and of the condensing enzyme were respectively about thirteen and sixty times higher in flight muscle than in fat body. It appears possible therefore that when a demand for energy by the flight muscle arises, fat in the fat body is hydrolysed by the active lipase present and the fatty acids are transported to the flight muscle where they are broken down to acetyl coenzyme A, and this, through the mediation of the condensing enzyme, enters the energy-yielding tricarboxylic acid cycle. The glycerol produced by the fat body lipase is presumably phosphorylated *in situ* (muscle being unable to do this), but the site of subsequent metabolism of the glycerophosphate formed is not yet clear.

VII. Protein and Amino Acid Metabolism

A. PROTEINS

The fat body contains labile protein reserves which may be seen as droplets, accumulating during periods of feeding and disappearing during starvation, as in experiments of Wigglesworth (1942, 1949) with *Aëdes* and *Drosophila*. The appearance of "albuminoid granules" in the fat body at the beginning of pupation was observed by an earlier generation of insect cytologists and their nature and origin was the subject of much speculation. They occur in various species, but to take the honey bee as an example, Oertel (1930) observed that soon after the larva was sealed in its cell, the nuclear walls in the fat body cells became indistinct and small albuminoid granules made their appearance in the cytoplasm close to the nucleus and migrated outwards towards the periphery of the cell increasing in size during the process. At a later stage some of the fat body cells broke down, the liberated contents presumably being used for histogenesis, for example, in the formation of the large and numerous thoracic muscles, while the granules in the remaining fat body cells largely disappeared. The blood of prepupae and pupae of various insects is often milky due to the presence of these granules released by the breakdown of fat body cells. The nitrogen content of the granules (8–9%) is much lower than it would be if they were composed entirely of protein (Ludwig, 1954) and the relatively large amount of non-nitrogenous material present may be lipid in nature, since the granules will stain with osmic acid. They also contain uric acid, but do not give a positive Feulgen reaction for nucleoprotein. It would seem that they represent reserve material possibly in the form of lipoprotein.

1. *Protein synthesis*

Some very interesting experiments of Shigematsu (1958a, 1960a, b) have indicated that the fat body synthesizes the globulins of the blood. The electrophoretic pattern of silkworm blood shows that the three main protein components are albumin, globulin I and globulin II. During development, not only does the total protein concentration in the blood change—rising from 2·6% to 7·26% during the fifth larval stadium—but the relative amounts of the components change, the level of the albumin remaining approximately constant while that of the globulins increases threefold. There is an increase of about 13 mg protein per individual per day during the latter half of the stadium, largely due to the appearance of new globulin, and Shigematsu's work showed that this new protein was synthesized in the fat body and secreted into the blood. He demonstrated

that the protein content of the fat body did not change *in vivo* during the period when the protein in the blood was increasing, but net synthesis of protein could be shown if the fat body was incubated *in vitro* with a casein hydrolysate and ATP. The amount of protein, in the tissue (500 mg of which was incubated), increased in an almost linear manner from 34 mg to 39 mg in 5 h, while during the same period about 4 mg of protein was liberated into the incubation medium. This would correspond to a secretion of about 12 mg protein per day per individual, a figure close to the observed increase of blood protein of 13 mg per day. Paper electrophoresis of the protein secreted by the fat body into the medium showed that two main components were present, globulin I and globulin II, whose mobilities were similar to those of the corresponding blood proteins, while albumin was absent. Confirmation that the fat body was synthesizing and secreting specific proteins was obtained by the use of radioactive amino acids as substrates, when the protein secreted into the medium after 5 h had a specific activity which was twenty times that of the tissue proteins. The incorporation of ^{14}C-amino acids was increased if the respiratory activity of the tissue was stimulated by addition of energy sources such as ATP, glucose, fructose, diphosphate etc., and largely stopped by inhibitors such as 2:4-dinitrophenol, cyanide and iodoacetate. It appeared that the potentialities for protein synthesis were greater than were allowed by the normal rate of penetration of amino acids into the intact cells, since homogenates incorporated amino acids at a rate 65% greater than that of intact tissue. Protein secretion by intact tissue was stimulated by calcium ions, and was higher when the medium was hypertonic. Amino acids were also incorporated into peptides which initially showed a higher specific activity than the protein fraction, but the relationship of these peptides to protein biosynthesis is not clear at present. Shigematsu also showed that incubation of glucose-^{14}C with fat body from silkworms also gave rise to labelled protein and free amino acids (in addition to other compounds) but it was not stated which amino acids became labelled.

If intracellular symbionts are present, they may contribute to amino acid synthesis in the fat body. Henry and Block (1962) fed glucose-^{14}C (G) and a vitamin mixture to *Blattella* with a normal symbiont flora, hydrolysed the tissues and showed the presence of fourteen labelled amino acids; but when the experiment was repeated with *Blattella* which had been freed from symbionts by aureomycin, no label was found in tyrosine, phenylalanine, isoleucine, valine, arginine and possibly threonine. The failure to synthesize these amino acids suggests, these workers remark, that the symbionts perform biochemical functions similar to

those of rumen micro-organisms. Henry and Block (1960) had shown previously that symbionts were necessary for the synthesis of cysteine and methionine from inorganic sulphate.

B. AMINO ACIDS

The fat body is an active site for the intermediary metabolism of amino acids. Using this tissue from *Schistocerca*, Kilby and Neville (1957) found that homogenates would catalyse transaminations between α-ketoglutarate and many amino acids—glycine, aspartate, leucine, valine, serine, threonine, phenylalanine, tyrosine, tryptophan, histidine, lysine, ornithine, arginine, cysteine, cystine and methionine. No synthesis of glutamate from α-ketoglutarate was observed with proline or hydroxyproline.

The two most active transamination reactions were:

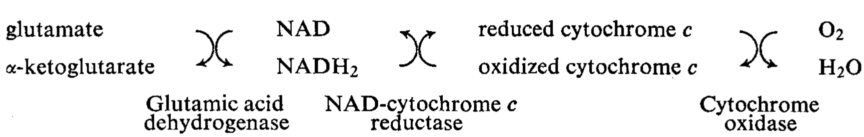
$$\alpha\text{-ketoglutarate} + \text{aspartate} \rightleftharpoons \text{glutamate} + \text{oxalacetate}$$
$$\alpha\text{-ketoglutarate} + \text{alanine} \rightleftharpoons \text{glutamate} + \text{pyruvate.}$$

The former was the more active, glutamate being produced at a rate of around 200μmole/g fat body (wet weight)/h at 37°C, while the second reaction proceeded at about half this rate and transaminations involving the other amino acids as amino group donors took place at approximately one-twentieth of the rate. These activities of transaminases involving glutamate are of the same order of magnitude as those found in rat liver and kidney (Awapara and Seale, 1952). The alanine/glutamate reaction was reversible, showed a broad pH-optimum between 6·5 and 8·0 and was pyridoxal phosphate dependent. All the transaminases were present in the mitochondrial fraction and the alanine/glutamate and aspartate/glutamate ones also occurred in the soluble fraction of fat body cell homogenates, and it is interesting to note that this distribution is the same as that found for the transaminases of rat liver (Hird and Rowsell, 1950). Glutamine can also serve as an amino group donor, the α-amino group being utilized rather than the amido one, so that the activity is of the Glutaminase II type rather than Glutaminase I, using the nomenclature of Errara (1949). A leucine/alanine transaminase is also present in *Schistocerca* fat body, and undoubtedly a further search would reveal others. A similar survey of transamination in the fat body of the larvae of *Calliphora erythrocephala* has been made (Desai and Kilby, 1958a), this species having been chosen as a holometabolous insect in contrast to the hemimetabolous locust, and the amino acid metabolism was found to be almost identical. A study of the distribution of the aspartate/glutamate

transaminase in the cockroach, *Periplaneta americana*, has been made by McAllen (1958) and the greatest activity (per mg wet weight tissue) was in the fat body and malpighian tubes, lower in flight muscle and gut, lowest in ventral nerve cord and absent from the blood. It would be unwise to generalize from one insect to another, however, since work with the pupae of the Hawk moth (*Celerio euphorbiae*) by Belzecka *et al.*, (1959) showed that aspartate/glutamate transaminase activity was ten times higher in muscle than in the fat body, and a very low activity was detectable in the blood. The alanine/glutamate enzyme occurs in various tissues of the silkworm, including the fat body and silkgland (Fukuda, 1957).

D-Amino acid oxidases, which act on the D forms of alanine, histidine, valine, leucine, glutamate and phenylalanine, have been found in the fat body of *Schistocerca* (Kilby and Neville, 1957) and of *Calliphora* (Desai and Kilby, 1958a). A very much weaker L-amino acid oxidase activity is present, and this may be due to a true L-amino acid oxidase but it could also result from a coupling of a transaminase and a glutamate cytochrome reductase. A broad survey of the amino acid oxidase activity of cockroach fat body towards a variety of amino acids has been given by Auclair (1959) and less extensive data for three other insect species. D-Amino acid oxidases are apparently absent from the milkweed bug *Oncopeltus*, and this may explain the occurrence of the unnatural isomer, D-alanine, in the blood of this insect (Auclair and Patton, 1950). Auclair discussed the possibility that the amino acid oxidase activity observed in cockroach fat body might be due to the activities of the intracellular symbionts which are present and concluded that this explanation could not be excluded. The use of aposymbiotic cockroaches would be valuable, but the presence of a similar pattern of amino acid oxidase activity in *Schistocerca* fat body, in which symbionts are absent (Coupland, 1957), indicated that in this species at least, the observations were due to true insect enzymes.

The presence of a glutamic acid dehydrogenase system in *Schistocerca* fat body has been shown by Kilby and Neville (1957) and can be expressed in a simplified form:



If cyanide is added to the homogenate to inhibit cytochrome oxidase, the oxidation of glutamate can be followed by observing the production of reduced cytochrome *c*, the system requiring NAD. This dehydrogenase

system, in conjunction with the various transaminases, affords a route for the overall oxidative deamination of various amino acids in the fat body:

$$\begin{array}{ccccc} \text{amino acid} & & \alpha\text{-ketoglutarate} & & NH_3 \\ & \rightleftarrows & & \rightleftarrows & \\ \text{keto acid} & & \text{glutamate} & & \tfrac{1}{2}O_2 \\ & \text{Transaminases} & & \text{Glutamic acid dehydrogenase system} & \end{array}$$

A similar glutamic dehydrogenase system linked to NAD and cytochrome *c* occurs in *Calliphora* larvae fat body (Desai and Kilby, 1958a).

Glutamine can be formed from glutamate by a *Schistocerca* fat body homogenate in the presence of ATP and magnesium ions. A rate of about 100μmoles/g tissue (wet weight)/h was observed by Kilby and Neville (1957).

Glycine and serine are interconvertable in many tissues (from vertebrates, plants, etc.), a reaction which involves an "active formate" (a folic acid derivative):

$$\begin{array}{l} CH_2NH_2 \\ | \\ COOH \end{array} + \text{"active formate"} \rightleftharpoons \begin{array}{l} CH_2OH \\ | \\ CH{-}NH_2 \\ | \\ COOH \end{array}$$

A similar interconversion is operative in insect fat body, since McEnroe and Forgash (1958) showed that fat body from *Periplaneta* incorporates formate-^{14}C into the β-position of serine, the rate of incorporation being increased over four times if glycine is also added. Clements (1959) incubated glycine-^{14}C (G) with locust fat body and found labelled serine in both the free amino acid fraction and in the proteins. The interesting observation was made by Clements that over half of the labelled glycine was completely oxidized to carbon dioxide and water by the fat body (leucine-^{14}C (G) behaving in a similar manner), yet when labelled glucose was incubated under the same conditions, less than one-third of it was completely metabolized. Locust muscle was found to be much less active in metabolizing amino acids completely, and the parallel was drawn with mammalian systems in which the liver can oxidize glycine at a high rate while muscle cannot do so at all.

McEnroe and Forgash also observed a high activity in proline after incubation of fat body with labelled formate, yet glutamate, which is a precursor of proline on the known pathway of synthesis, had a much lower activity than had proline. Possible explanations offered were that the pathway of proline synthesis is different in insects, or that the turnover of glutamate might have been so high that by the end of the 5 h incubation period much of the active glutamate might have been converted

into proline or metabolized. Further experiments should readily resolve this point. The same workers found that methionine was not labelled to any significant extent, suggesting that there was no active reduction of formate to methionine methyl groups. This may be associated with the necessity of choline being present in the diet either as an absolute requirement or for optimal growth.

Some of the sulphur-containing amino acids can take part in detoxification mechanisms. Cohen and Smith (1962) fed *p*-nitrobenzylchloride to *Schistocerca* and observed that it was excreted as *p*-nitrobenzylglutathione, *p*-nitrobenzylcysteine and *p*-nitrobenzoic acid. The first part of the detoxification can take place in the fat body since homogenates were observed to convert *p*-nitrobenzylchloride into p-nitrobenzylglutathione, the process being enhanced by addition of glutathione. The product could be converted into *p*-nitrobenzylcysteine by homogenates of Malpighian tubes or gut.

An amino oxidase has been found in various tissues of *Periplaneta* by Blaschko *et al.* (1961). It was most active in the Malpighian tubes but some activity was detectable in the fat body and other organs by qualitative tests using substrates such as β-phenylethylamine or *iso*amylamine. Its function may be to remove pharmacologically active amines, since compounds such as catechol amines and 5-hydroxytryptamine are known to occur in insects.

VIII. PURINES AND PTERIDINES

A. URIC ACID

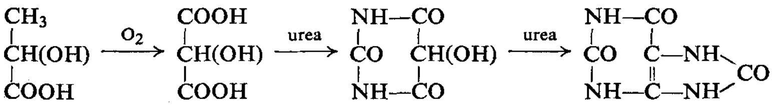
Fabre, as long ago as 1856, showed that the fat body cells of some insects contain particles which give the murexide reaction. Since then other workers have described the deposition of urates in the fat bodies of various insects (for a review of early work see Hollande, 1914). The presence of special cells in the fat body in which uric acid accumulated was noted, and around the turn of the century the theory was proposed that here was an example of storage excretion, Cuenot describing the urate cells of Orthoptera as "les reins d'accumulation". In the cockroach, for instance, the urate cells appear in the fat body of the embryo and uric acid accumulates in them throughout the life of the insect, and no uric acid has been found in the Malpighian tubes. Other examples are known of uric acid accumulating, and sometimes crystallizing out in the living fat body cells in insects in which the Malpighian tubes are functional and where there is no obvious anatomical barrier to normal urate excretion. In the larvae of bees, the Malpighian tubes end blindly and do not establish connection

with the gut until late pupal life or just after emergence of the adult bee; thus the larvae store uric acid as the end product of nitrogen metabolism, in the fat body until it can be transferred and excreted (see Wigglesworth, 1953). The cockroach normally accumulates uric acid harmlessly in the fat body during its life, but an insect which uses internal storage to dispose of its nitrogenous waste might be expected to get into difficulties under conditions where these become excessive. This was found to occur in some experiments of Haydak (1953) in which American cockroaches were fed on diets containing different amounts of protein. With low protein diets (11 % or below) the fat body was meagre or almost absent, normal development was seen on a 22–24% protein diet, but feeding with 79–91 % protein led to a greatly enlarged fat body which completely filled the abdominal cavity, compressing the other organs and distending the abdomen. The fat body was filled with white deposits of uric acid which occurred almost everywhere in the insect. This very high protein diet reduced the life span of the cockroaches and death may well have been due to their being literally choked by their own wastes. If these insects were transferred on to a low protein diet, much of the excessive uric acid deposits disappeared and there was a loss of body weight of 7–23%. It would be interesting to know the fate of this uric acid, since if uricase is present and able to break it down into allantoin, then it is not clear why uric acid should be accumulated in cockroaches on a normal diet.

The question arises of whether uric acid is formed exterior to the fat body and transported there for storage, or whether products of nitrogen metabolism are carried to the fat body in order to be converted into uric acid as an innocuous end product. Hollande (1914) injected sodium and ammonium urates into *Vanessa* larvae at a time when urate granules were just appearing in their fat bodies, but observed no increase in granule size compared with those in control insects. Uric acid granules did, however, appear in the Malpighian tubes. In experiments of Wigglesworth (1942) in which mosquito larvae were starved, uric acid was observed to appear in the fat body in greater amounts than could have been derived from the protein originally present there. Feeding with uric acid did not increase the deposits, and the evidence, while not conclusive, was considered to make it likely that the uric acid was made from products of deamination of amino acids or proteins brought to the fat body from elsewhere. Since the fat body has recently been shown to be able to synthesize uric acid *in vitro* (see below), it appears probable that the fat body is the actual site of uric acid formation, utilizing nitrogen in the form of glycine, aspartic acid and amide-nitrogen of glutamine, if the same pathways of biosynthesis is assumed to occur in insects as in higher animals.

B. PURINE BIOSYNTHESIS

In 1902, Wiener suggested that uric acid arose *in vivo* from the condensation of a dibasic three-carbon aliphatic acid and two molecules of urea. Tartronic acid was thought to arise from lactic acid and to give rise to uric acid as follows:



Tartronic acid Dialuric acid Uric acid

The scheme showed close analogies with pathways of purine synthesis carried out in the laboratory by Emil Fischer and associates, and it undoubtedly appeared very reasonable in context of its time, when the subtlety and sophistication of biosynthetic pathways was unsuspected. It led Liefert (1935) to investigate the biosynthesis of uric acid *in vitro* by fat body and other tissues from the larvae of the moth *Antheraea pernyi*, using malonic acid, urea and other simple nitrogenous materials as substrates. Her results appeared to support this scheme. In the two experiments in which fat body was incubated for $3\frac{1}{2}$ h with malonate and urea, increases of 84% and 107% in uric acid content were found, but if the urea was omitted, the increase was only about 16%. Other tissues gave similar results. Urea could be replaced by ammonium carbonate. Brighenti (1941) obtained an increase of uric acid in silkworm blood and excreta after injection of malonate, tartrate or ammonium salts, but not if urea was used. In recent years, a pathway for purine biosynthesis has been elucidated by Buchanan, Greenberg and others which has been found to be operative in many different animal tissues. This scheme, which for convenience may be termed the carboxamide ribotide pathway, involves the participation of glycine, formate, ATP, ribose-5-phosphate, glutamine, aspartate, etc., and differs completely from the hypothetical urea–malonate route of Wiener. In the light of these developments, the pathway of uric acid synthesis in insect fat body has been reinvestigated but no evidence in support of the malonate–urea scheme has been obtained. Anderson and Patton (1955) incubated fat body extracts of *Prodenia eridania* and *Tenebrio molitor* with various suspected uricogenic substrates. Increased formation of uric acid was given when other purines were used, but urea had no effect and malonate actually inhibited the system. Monoethyl oxalacetate gave a small increase in uric acid, but other non-cyclic substrates were without significant effect. Heller and Jezewska

(1959) have suggested that this failure to demonstrate any influence on uric acid synthesis by addition of compounds known to be precursors of the purine ring formed by the carboxamide ribotide pathway (e.g. glycine, formate, glutamine), may have been due to the fact that these precursors were used singly instead of the whole set being added simultaneously. They tested this idea and showed that if all the precursors (about a dozen) were used together, increases in uric acid of 40–500% were obtained as compared with controls. The pathway may be considered to begin with ribose-5-phosphate, and omission of this single component from the set reduced the increase of uric acid to about a third or a fifth of that obtained when it was also present. Heller and Jezewska used homogenates of fat body and gut from larvae or pupae of *Anthereae pernyi* and were able to free their preparations from much of the uric acid originally present by low temperature alcoholic precipitation. Their incubation mixture showed a maximum activity at pH 7·4 but was inactive at pH 6·6. If precursors sufficient for the carboxamide ribotide synthesis were added, then the presence of imidazole compounds could be shown chromatographically. While this work was in progress, Desai and Kilby (1958b), using fat body from *Calliphora* and *Telea polyphemus*, also found no increase in uric acid after incubation with malonate and urea, but showed a large increase with 4-amino-5-imidazole carboxamide. The ribotide of this compound is on the accepted route of purine biosynthesis, and the observation supported the idea that the carboxamide ribotide pathway was also used by insects. Additional confirmation has been given by McEnroe and Forgash (1957) who showed that ^{14}C-formate injected into American cockroaches gave rise to a uric acid in the fat body which was labelled in the 2 and 8 positions, a result which would be expected from the incorporation of formate in the carboxamide ribotide pathway. A similar result was obtained using fat body *in vitro* (McEnroe and Forgash, 1958). The route of purine biosynthesis therefore appears to be the same in insects as in other organisms, and the reasons for the increases or apparent increases in uric acid observed by Liefert with urea and malonate remain inexplicable.

C. PURINE METABOLISM

1. *Adenase, guanase and xanthine oxidase*

When specific tests have been made on fat body tissue for the presence of adenase, guanase and xanthine oxidase, the results have usually been positive (Table IV). Older work in which homogenates of whole insects were used have led to a number of reports of the apparent absence of some

of these enzymes from certain insects, but this may be due to the less sensitive experimental technique. The fat body is thus able to make uric acid from less oxidized purines, and this has been demonstrated *in vitro* by various workers; for instance, Anderson and Patton (1955) showed that fat body from the larvae of *Prodenia* and *Tenebrio* formed uric acid when incubated with adenine, guanine, hypoxanthine and xanthine. Adenase

TABLE IV

Occurrence of three enzymes in insect fat bodies

Species	Adenase	Guanase	Xanthine oxidase	Reference
LEPIDOPTERA				
Antheraea pernyi			+	Liefert (1935)
Prodenia eridania	+	+	+	Anderson and Patton (1955)
COLEOPTERA				
Tenebrio molitor	+	+	+	Anderson and Patton (1955)
			+	Irzykiewicz (1955)
DIPTERA				
Calliphora	+	+	+	Desai and Kilby (1958a)
ORTHOPTERA				
Leucophaea maderae		+	+	Liza and Ludwig (1959)
Periplaneta americana			+	Anderson (1953)

and guanase may serve to remove adenine and guanine arising from the degradation of nucleic acid, etc. If purine synthesis *de novo* takes the same pathway as in pigeon liver, etc., inosine-5-phosphate would be formed. This might be broken down to give hypoxanthine which could then be oxidized to uric acid by the xanthine oxidase present, which would thus be an essential enzyme in the pathway leading to uric acid.

The fat body of *Tenebrio* larvae makes up about 10% of the body weight, but was shown by Irzykiewicz (1955) to contain over a third of the xanthine oxidase of the insect. The mid-gut showed a similar high specific activity. The properties of the crude enzyme suggested that it resembles the xanthine oxidase of milk or liver in being a metal-containing dehydrogenase. A dialysed preparation showed activity only in the presence of a hydrogen acceptor such as methylene blue or, less efficiently, NAD plus pyruvate, which gave only 17% of the activity of the system containing methylene blue. The insect enzyme was similar to the mammalian one in

being inhibited by cyanide, 2-amino-4-hydroxy-6-formylpteridine and excess xanthine. High concentrations of adenase, guanase and xanthine oxidase are present in the fat bodies of *Periplaneta* and *Prodenia*, with lower activities in the gut and other tissues (Anderson, 1953).

Although uric acid is the major end product of nitrogen metabolism in most insects, a number of other compounds have been detected, such as

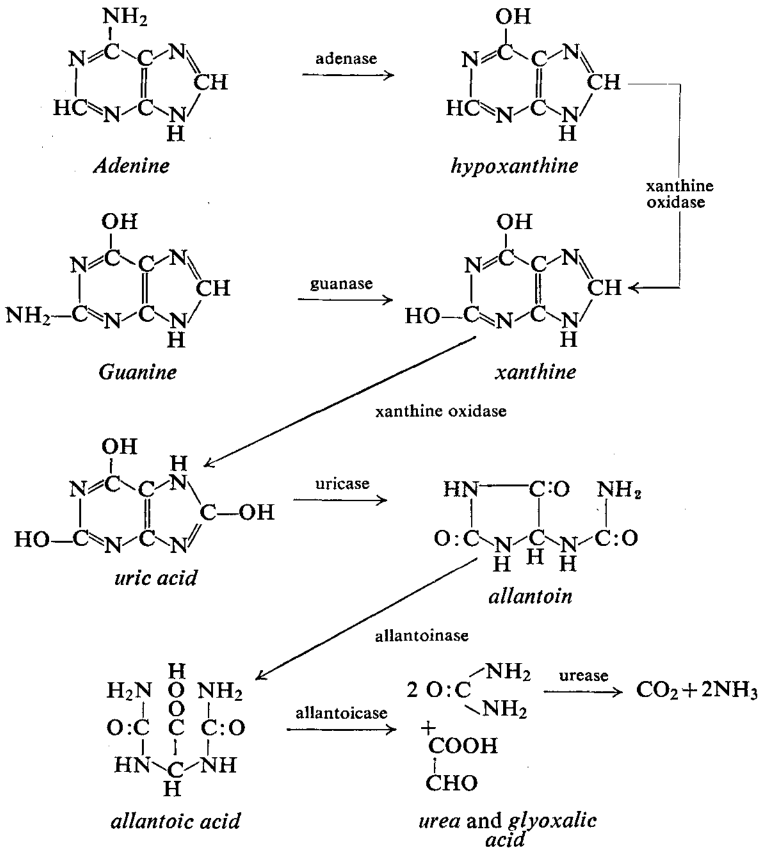


FIG. 5. Degradation of purines.

hypoxanthine and xanthine in the excreta from *Melophagus ovinus* (Nelson, 1958), allantoin from aquatic *Hemiptera* (Poisson and Razet, 1953), allantoic acid from some Lepidoptera (Razet, 1954, 1956) urea from *Rodnius* (Wigglesworth, 1931), while ammonia is excreted in relatively large amounts by blowfly larvae (Brown, 1936). All these compounds are intermediates in the metabolic scheme shown in Fig. 5 and might (but not

necessarily) arise by the metabolism of purines stopping short of uric acid or proceeding beyond it.

2. *Uricase*

This has been shown to occur in the fat bodies of various insects, e.g. *Antheraea* (Liefert, 1935), *Leucophaea* (Lisa and Ludwig, 1959), etc. Desai and Kilby (1958a) found that the uricase activity in the larval fat body of *Calliphora* decreased during larval life and disappeared by the time of pupation (Table V), while uric acid could not be detected in the fat body

TABLE V

Variation of uricase activity in *Calliphora* larvae with age

Age of larva in h	Uricase activity in μg uric acid oxidized per h per gm fat body
45	654
67	813
100	160
120	144
145	50
160	0

(Pupation usually occurs at age of 160–180 h)

before the 70th h of life. A similar result had been obtained by Brown (1938a) using whole homogenates of the larvae of *Lucilia*, in which he found that the uricase activity disappeared abruptly when the larvae left the meat in order to become prepupae and reappeared suddenly again on emergence of the adult flies. Allantoin is the major end-product of purine metabolism in these maggots during the period while uricase is active.

3. *Allantoinase*

Allantoin is hydrolysed to allantoic acid in the presence of this enzyme which has been found in a number of insects, but appears to be absent from others. In some species (e.g. *Blaberus* and in the larvae of *Saturnia* and *Bombyx*) the highest allantoinase activity is found in the fat body, but in others (*Calliphora*, *Gryllus*) the activity here is low and most of the enzyme is located in the Malpighian tubes (Razet, 1953). Some caterpillars excrete uric acid and allantoic acid in almost equal amounts, but the adult insects excrete mainly uric acid (Razet, 1956). At present, it is

difficult to discern any consistent pattern in the insect kingdom for the occurrence or absence of allantoinase, or in its location if present. A suggestion of Rocco (1936) that a herbivorous way of life is associated with uric acid excretion and a carnivorous one with excretion of allantoin or allantoic acid does not appear to be valid.

4. *Allantoicase and the origin of urea*

Florkin (1949) has suggested that the evolution of purine metabolism has operated by the curtailing of the enzymic chain of uricolysis. In marine invertebrates, uric acid may be degraded to ammonia by the successive action of uricase, allantoinase, allantoicase and urease, but during progress up the evolutionary scale, the final links in the chain have been lost in succession, until in the primates, all four are lacking. Insects are in the intermediate position of often possessing uricase and allantoinase, while urease has not been detected and the status of allantoicase is uncertain. Florkin and Duchateau (1943) were unable to detect allantoicase in whole homogenate from five insects (*Hydrophilus*, *Tenebrio*, *Dytiscus*, *Aeschna* and *Limnophilus*), but Manunta (1948) has reported that it occurs in silkworms but only at certain developmental stages and under given conditions, e.g. the activity was said to increase in larval tissues during starvation. If the enzyme is normally absent in insects or present only at a very low level of activity, it would appear that allantoic acid would not be split into glyoxylic acid and urea; since the latter is often found as a minor constituent of insect excreta and has been detected in insect bloods (cf. Buck, 1953), its origin would still be unknown. A fairly active arginase has been found in the fat body of *Schistocerca* which produced ornithine and urea from arginine at a rate of 610 μmoles/g fat body (wet weight)/h at 37° (Kilby and Neville, 1957). The enzyme has also been found in the fat body of the larvae and adults of the moth *Celerio euphorbiae*, and was shown to have a pH optimum of 9·5, which is similar to that of the mammalian enzyme (Szarkowska and Porembska, 1959). Desai and Kilby (1958a) could not detect arginase, however, in *Calliphora* fat body. The operation of the Krebs-Henseleit urea cycle has yet to be demonstrated in insect tissue, and the function of the active arginase when present is obscure. Perhaps this may turn out to be concerned more with the formation of ornithine for some as yet unknown purpose, rather than primarily with urea synthesis. Urea in the excreta may well be derived largely from the diet, since Brown (1938b) found that sterile *Lucilia* maggots did not produce urea when fed on a sterile or contaminated synthetic diet, but did so if fed on contaminated meat. The urea excreted by Rhodnius may originate in the blood taken as food (Wigglesworth, 1931).

The larvae of blowflies produce large amounts of ammonia but its origin is uncertain. Production from urea can be excluded owing to the absence of urease. A powerful anaerobic deaminase which acted on peptides but not free amino acids was found by Brown and Farber (1936) in the gut of *Lucilia* and *Calliphora*, while an adenosine deaminase investigated by Lennox (1941) may be an important contributory cause of the ammonia production. Adenosine deaminase was found in the fat body of *Calliphora* by Desai and Kilby (1958a) but the fat body may possibly not play a very important part in ammonia production by this enzyme since in *Lucilia* the greatest activity is present in the gut and Malpighian tubes.

5. *Uric acid as a nitrogen store*

Ludwig (1954) has suggested that the uric acid associated with the "albuminoid granules" formed in some fat bodies and liberated during pupation, may be a nitrogen reserve, to be utilized during histogenesis. General utilization of the purine nitrogen of uric acid would presumably require the presence of the full complement of enzymes for the complete degradation of the purine ring and the release of the nitrogen as amino groups. An alternative possibility, suggested by Ross (1959), is that the uric acid might be reduced to hypoxanthine by xanthine oxidase acting in reverse, possibly with some aldehyde acting as a hydrogen donor, and the hypoxanthine would then be available to supply the purines required for nucleoprotein synthesis. This idea would fit in with the observations made on the Japanese beetle, in which uricase is absent after the early prepural stage and yet uric acid decreases in the late pupal period at a time when there is a sudden rise in xanthine oxidase activity. It would be interesting to test these speculations by injection of labelled uric acid at an appropriate stage of the life history, and see if any of the nitrogen was incorporated in other compounds and, if so, whether it was restricted to other purine compounds or generally utilized.

The intracellular symbiotic bacteria which occur in the fat body of the cockroach have been isolated and cultured by Keller (1950) who was able to grow them on a simple agar medium containing only 0·2% uric acid and 0·3% sodium chloride (pH 7·6). Growth took place at the expense of the uric acid and some unidentified acidic product of metabolism was formed (oxalic acid ?). The bacterium was named *Rhizobium uricophilum*, since its morphological and physiological properties appeared similar to *R. leguminosarum*, the symbiont of Leguminous plants. Both could utilize uric acid but not urea, would reduce nitrate to nitrite, had similar abilities to metabolize some sugars and not others, etc. If these bacteria utilize uric acid *in vivo*, then a possible route exists for the

remobilization of the nitrogen locked up in uric acid. A more vital role of the symbionts, however, is probably to supply the host with essential growth factors; female cockroaches, for instance, which have been made asymbiotic show degeneration of the ovaries.

D. PTERIDINES

A number of these compounds occur fairly widely in the insect kingdom, where their most obvious function is to serve as red, white or yellow pigments, as in wing scales of some Lepidoptera, in the hypodermis of Hymenoptera, etc., and especially as eye pigments, and there are also indications that the pteridines may have other functions in insect physiology (see Gilmour, 1961). There is no information concerning the site of their biosynthesis in the insect, but the fat body may turn out to be one location, since this organ in the milkweed bug, *Oncopeltus fasciatus*, has been shown to contain about 20% of the total *iso*-xanthopterin and 30% of the xanthopterin found in the insect (Hudson *et al.*, 1959). The pteridines may be formed from purines, since feeding ^{14}C-purines to caterpillars gives rise to labelled pteridines in the wing scales of the butterflies after pupation. Glassman and Mitchell (1959) have shown that xanthine dehydrogenase purified from wild-type *Drosophila* would oxidize xanthopterin to leucopterin and 2-amino-4-hydroxypteridine to *iso*-xanthopterin as well as oxidizing hypoxanthine to uric acid through xanthine. They found also that certain mutants, *ry* and ry^2 (rosy eye colour) and *Ma-l* (maroon-like eye colour) showed a complete absence of xanthine dehydrogenase, and as a result these insects contained a biochemical excess of hypoxanthine and 2-amino-4-hydroxypteridine and deficiencies of uric acid and *iso*-xanthopterin. An explanation is thus afforded for the earlier and elegant work of Hadorn and Schwinck (1956) who demonstrated that eye disks of the mutant ry^2 produced normal amounts of pigment when transplanted into wild-type hosts, but pigment production in wild-type eye disks was inhibited if transplanted into mutant hosts. A factor ("ry^+ – Stoff") was postulated as being necessary for pigment production and which was absent in the mutants, but could be supplied by implanting larval fat bodies and Malpighian tubes from wild type *Drosophila*. It now appears that the factor is xanthine dehydrogenase. Ursprung and Hadorn (1961) have examined the distribution of this enzyme in different tissues of wild type *Drosophila* by incubating the homogenized tissue with 2-amino-4-hydroxy-pteridine as substrate and methylene blue as hydrogen acceptor and estimating the *iso*-xanthopterin formed after separation by paper chromatography. They found a

high xanthine dehydrogenase activity in the fat body, and a much smaller one in the Malpighian tubes, and the efficiency of implanted fat body in Hadorn and Schwinck's experiments is thus explicable. The presence of the enzyme in the fat body may also explain why 2-amino-4-hydroxy-peterine was absent in *Oncopeltus* fat body although it was present in mid-gut (Hudson *et al.*, 1959). The absence of *iso*-xanthopterin and uric acid in *white* and *brown Drosophila* mutants has a different explanation, since these mutants contain normal amounts of xanthine dehydrogenase, and the deficiency of *iso*-xanthopterin appears to result from a lack of its precursor, 2-amino-4-hydroxpteridine which is excreted, while the absence of uric acid is associated with a higher activity of uricase than that found in the wild-type (Ursprung, 1961). The xanthine oxidase of the silk-worm fat body has been purified one hundred-fold (Hayashi, 1962), and shown to have similar properties to the *Drosophila* enzyme, dehydrogenating hypothanthine, xanthine and 2-amino-4-hydroxypteridine in the presence of methylene blue or triphenyltetrazolium chloride.

IX. Pigments in the Fat Body

The literature on insect pigments in general has been reviewed by Cromartie (1959). Much of the published work has been primarily concerned with the isolation, purification and determination of the structure of the pigments, rather than to their exact location. A number have, however, been reported in various fat bodies.

A. CAROTENE

Some fat bodies are quite colourless, as in *Calliphora* larvae, but more frequently they have a yellowish tint which may be correlated with the abundance of carotene in their diet; the colour is yellowish white in cranefly and wasp larvae, but deep yellow in locusts and in some caterpillars (Buys, 1924). Goodwin and Srisukh (1949) found that the yellow colour of the fat bodies of *Schistocerca* and *Locusta* is due to β-carotene and that this is the only carotenoid pigment present, although astaxanthin occurs elsewhere in the insect. The carotene must be derived from the food since insects are unable to synthesize it, and it can be regarded as storage material in the fat body and elsewhere. The female locust requires considerable amounts of carotene during egg formation; a pod of eggs contains about 20 μg, an amount of the same order as the total in the whole body of the male or a female without eggs (Goodwin, 1952). A selective absorption or metabolism of carotenoids from the food must occur, since the xanthophylls which accompany carotene in the plant are not found in the insect blood or fat body.

B. HAEMOGLOBIN AND BILE-TYPE PIGMENTS

Haemoglobin occurs only in a few insect species, for example, in the blood of certain Chironomid midge larvae, and in the accessory genital glands of *Macrocrixa*; in *Gastrophilus* larvae it is present in the fat body, but only during that part of the life cycle which the larvae spends attached parasitically to the stomach wall of the horse. During the earlier part of this stage, the haemoglobin is distributed throughout the fat body, but later it becomes more concentrated and localized in special tracheal cells, which develop from undifferentiated fat body cells during the first larval instar. The function of these cells, which make up the tracheal organ, appears to be to enable the larva to make better use of the intermittent air supply which reaches it in the form of bubbles trapped in the horse's food (Keilin and Wang, 1946). Levenbook (1951) has made a study of these tracheal cells, which appear to be similar histologically (apart from the haemoglobin and intracellular tracheoles) to the ordinary fat body cells in the same insect, and indeed, the two types merge imperceptibly into one another. The *Gastrophilus* haemoglobin was obtained crystalline by Keilin and Wang, and its properties recorded. It has a molecular weight half that of mammalian haemoglobin and, in consequence, only 2 haems per molecule instead of 4.

Bilirubin and biliverdin, which accumulate in the fat body of *Chironomus* larvae, are formed by the degradation of some of the haemoglobin in the blood. On the other hand, the blood-sucking bug *Rhodnius* does not appear to use the fat body to store biliverdin, but accumulates it in the pericardial cells (Wigglesworth, 1943).

Metcalf (1945) has studied the metabolism of chlorophyll in the squash bug, *Anasa tristis*, and has shown that the apple-green colour of the fat body is due to the presence of a tetrapyrrolic compound derived from chlorophyll and analogous to the biliverdin from haemoglobin.

C. OMMOCHROMES

Certain eye pigments in insects belong to a group of compounds termed ommochromes, which are derived biosynthetically from tryptophan by a series of enzymically controlled reactions. Pigment formation may fail in certain mutants owing to the absence of any one of the necessary enzymes. A combination of genetical and microchemical studies on such mutants gave the first indication that genes express themselves phenotypically by controlling the synthesis of enzymes. A recent review of the genetical aspects has been made by Ziegler (1961), and in the present article only a brief mention will be made of this fascinating and complex

field, which extends far beyond the scope of the biochemistry of the fat body.

The pathway of biosynthesis of xanthommatin is shown in Fig. 6. Tryptophan is oxidized in a series of stages to hydroxy-kynurenine, and two molecules of this then undergo oxidative condensation with the elimi-

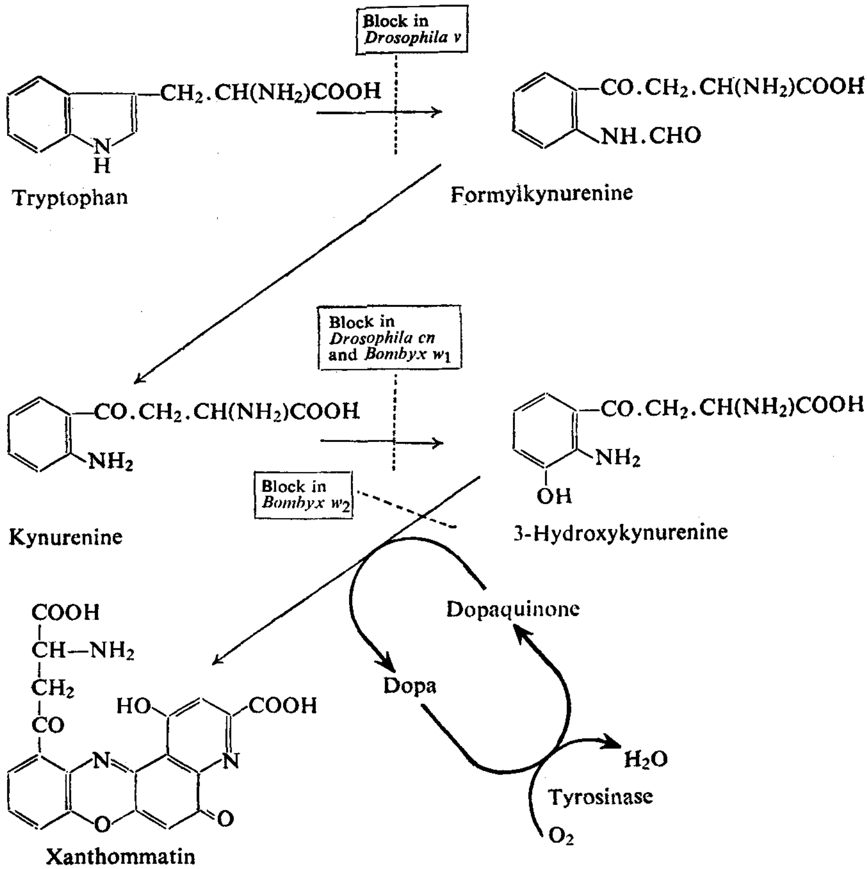


FIG. 6. Biosynthesis of xanthommatin.

nation of a molecule of ammonia and the loss of eight hydrogen atoms to give xanthommatin. Dopaquinone acts as the hydrogen acceptor in this last stage and is reduced to dopa, but this can be re-oxidized back to dopaquinone by tyrosinase and oxygen, so that only catalytic amounts are required.

Kynurenine accumulates in the fat body of the wild-type *Drosophila*

towards the time of pupation and can be observed as light blue fluorescent globules by fluorescent microscopy (Rizki, 1961). This fluorescent material is absent from the fat body of the *vermillion* (v) mutant of *Drosophila*, which lacks tryptophan peroxidase and is therefore unable to perform the first stage in the reaction chain, i.e., the conversion of tryptophan to formylkynurenine. As would be expected, the concentration of free tryptophan is higher in the tissues of the mutant than in the wild type. Kynurenine is normally utilized during pupation for pigment formation, but in the *cinnabar* (cn) mutant, kynurenine accumulates because the enzyme which catalyses its conversion to 3-hydroxy-kynurenine is missing. Kikkawa (1953) reported a somewhat similar effect in *Bombyx*, where the *white-1* (W_1) mutant accumulates kynurenine (up to 0·3–0·5 mg/g wet weight in the pupa) while mutant *white-2* (W_2) accumulates 3-hydroxy-kynurenine. In *red cell* mutant of *Drosophila*, pigment formation takes place in some cells of the anterior fat masses, but during the pupal stage these larval fat body cells separate from each other and rearrange so that they become located mainly in the head and thorax of the adult.

Rizki observed that the fluorescent material in Drosophila fat body first appears at a larval age of 96 $\pm$ 2 h as a light blue fluorescence around the nucleus, and within a few hours the anterior fat body is sharply differentiated from the remaining part. He remarks: "The present example offers desirable conditions for further study, for here a specific gene locus, v, is involved in the elaboration of a particular biochemical, kynurenine, which accumulates near the nuclear membrane in a group of differentiated cells during a specific period of development."

A red ommochrome pigment appears in the cells of the epidermis and fat body of the larvae of *Cerula vinula* about 5 days before the pupal moult, causing the green caterpillars to turn a deep red. Büchmann (1959) has shown that the formation of this pigment is controlled by α-ecdyson, the hormone produced by the prothoracic gland, and that colour change can be prevented by trying a suitable ligature. Injection of α-ecdyson into the green ligatured abdomens will stimulate pigmentation at a dose level around 66 *Caliphora* units, a very much lower dose than is required to bring about a pupal moult (3300–6600 units).

D. ANTHOCYANIN

There are a number of cases in which the presence of pigment in the fat body may be regarded as excretion storage. One such instance is afforded by the larva of the beetle *Cionus olens* which feeds on the flowers of

Verbascum, and its fat body contains granules of purple pigment which appear to be identical with the anthocyanin in the stamens of the flowers. The fluid in the gut and blood is without the characteristic purple colour, indicating that the anthocyanin is absorbed and transported in the leuco form. During metamorphosis, the fat body disintegrates and the pigmented cells are ingested by phagocytes and the pigment changes from purple through blue and red to colourless and its presence in the phagocytes in the leuco form can be demonstrated chemically. The observation of the pigment could serve to indicate the pH or O/R state of the tissue (Hollande, 1913).

E. Other Pigments

An interesting example of fat body pigmentation has been described by Hinton (1958). The cuticle of the larva of *Thaumalea verralli* is transparent and the colour pattern of the dorsal surface is determined by the distribution of pigmented and unpigmented cells of the peripheral fat body. During pupation, the cells of the fat body dissociate, the pigmented cells (chromatocytes) migrate over considerable distances (for instance, from the dorsal to the ventral side of the abdomen) and take up a different arrangement, forming the new colour pattern visible in the adult. The chemical nature of the pigment in the chromatocytes is unknown, but melanin is excluded, because the pigment can be oxidized and reduced by mild reagents.

Adult beetles of the family *Lampyridae* very frequently have a rose colouration of certain areas of the pronotal disc, and this has been shown by Metcalf (1943) to be due to a pigment which occurs in the fat body and male gonads. The compound, which was named lampyrine, is characterized by a beautiful rose-red fluorescence under ultra-violet light and has been isolated in crystalline form, but its structure is unknown. The presence of the pigment appears to be almost a biochemical characteristic of the family *Lampyridae*, since Metcalf was able to detect it in forty-three out of forty-five species.

X. Conclusion

The fat body has two well defined functions—storage and intermediary metabolism. The main purpose of the larval stages of some insects, particularly holometabolous ones, appears to be the accumulation of an adequate supply of all the substrates which will be required for the histogenesis of the adult form, with the result that the larger part of the mature larva may consist of a fat body crammed with these materials, especially fat, carbohydrate and protein. Other useful materials, such as carotene,

vitamins, pteridines, etc., may also be present, or compounds such as anthocyanin which almost certainly represents a stored excretion product of doubtful future value to the insect. The status of stored uric acid is a little uncertain. It has long been considered a waste product, stored harmlessly, but there is the possibility that a part at least might serve as a nitrogen reserve (uric acid contains over 33% nitrogen); but, as yet, the enzymes which would be necessary to tap this reserve and liberate the nitrogen in a useful form, have not been demonstrated.

Various types of metabolic activity are operative in the fat body. Enzyme systems are present for the synthesis to reserve materials from small molecules brought by the blood, and a good deal of interconversion is possible; the experiments of Clements showed that incubation of the fat body with labelled amino acid, acetate or glucose gave rise to labelled trehalose, glycogen, fat and protein. There are times when the mobilization of reserves becomes necessary, as in a period of starvation or to meet the energy requirements of prolonged flight; some of the necessary enzymes, e.g. lipase and phosphorylase, have been detected, but there appears to have been little or no investigation of intracellular proteinases. The tissue also serves as the site for the biosynthesis of compounds which are liberated into the blood, blood trehalose and blood globulin being good examples, and kynurenine is probably another one. Future work may show whether other characteristic insect blood constituents, such as citrate and glycerol, are derived from the metabolism of fat body cells. Finally, reactions can be carried out which are in the nature of "detoxification", although some examples investigated are rather artificial (e.g., *p*-nitrobenzylchloride and 4-methyl umbelliferone); the formation of uric acid as the end product of nitrogen metabolism may be regarded as a method of rendering ammonia innocuous, but it is not clear yet whether the fat body is the only or even the main site for uric acid synthesis, or whether the uric acid is transported from other tissues.

The composition of insect blood can vary between much wider limits than are permissible in mammals, but homeostatic regulation is effected by tissues like the fat body, Malpighian tubes, anal papillae, etc. The level of blood glucose is relatively constant, and a big rise after absorption through the gut of digested carbohydrate is prevented by the rapid conversion of glucose into trehalose or glycogen by the fat body. A sharp fall in the blood trehalose concentration during periods of rapid utilization, as in flight, is prevented by an almost as rapid synthesis and release of trehalose by the fat body.

There is not yet sufficient data available to justify much speculation upon the physiological factors which regulate the metabolic activity of

the fat body, although it is quite clear that hormonal action is involved. The fat body responds rapidly to stimulation by the moulting hormone, α-ecdyson, Wigglesworth (1959) reporting "that within six hours after feeding, or injection of ecdyson, there is a detectable increase in ribonucleic acid around the nuclei of these cells. Within twenty-four hours after feeding, the nucleoli are greatly enlarged and lobulated; much nucleic acid is accumulating; the mitochondria appear to be increasing in number; there are many filamentous forms which seem to be radiating from the nucleus and many of them are branching." This activity is the prelude to an increase in protein synthesis, possibly brought about by stimulation of the biosynthesis of enzymes and respiratory carriers.

As recently as 1951 it was possible to write "The physiological processes associated with the fat body are obscure" (Imms). Wigglesworth, in 1953, could say that its most obvious function was the storage of reserve materials, but it might have other functions in intermediary metabolism. Progress has been rapid during the past decade, and although the present review is by no means exhaustive in its treatment, it will be seen that there is now adequate evidence that the fat body is one of the major sites for intermediary metabolism involving all the more important substrates. The analogy which has repeatedly been drawn between the fat body and the mammalian liver is thus not without foundation, but the analogy must clearly not be pressed too far. However, the biochemical processes which occur in these two tissues are often very similar, and it is not easy to find important differences; for instance, although the amino acid level in insect blood is very much higher than that in mammalian blood, this does not appear to be reflected in any unusual pattern of amino acid metabolism in the fat body when compared with liver. The formation of trehalose and its breakdown may be quoted as one of the few examples of biochemical processes which have not yet been demonstrated in liver.

Much work still remains to be done on the biochemistry of the fat body, and possibly unique features and interesting differences will be revealed as the gaps in our knowledge are closed and fine detail elucidated.

References

Anderson, A. D. (1953). "Uric acid synthesis in insects". Ph.D dissertation, Cornell University.

Anderson, A. D. and Patton, R. L. (1955). *J. exp. Zool.* **128**, 443–451. *In vitro* studies of uric acid synthesis in insects.

Auclair, J. L. (1959). *J. insect Physiol.* **3**, 57–62. Amino acid oxidase activity in the fat body and Malpighian tubes of some insects.

Auclair, J. L. and Patton, R. L. (1950). *Rev. canad. Biol.* **9**, 3–8. On the occurrence of D-alanine in the haemolymph of the milkweed bug, *Oncopeltus fasciatus.*

Awapara, J. and Seale, B. (1952). *J. biol. Chem.* **194**, 497–502. Distribution of transaminases in rat organs.

Bade, M. L. (1962). *Biochem. J.* **83**, 478–482. Metabolic conversions during pupation of the cecropia silkworm. II—Tests for the operation of the glyoxalate cycle.

Bade, M. L. and Wyatt, G. R. (1962). *Biochem. J.* **83**, 470–478. Metabolic conversions during pupation of the cecropia silkworm. I—Deposition and utilisation of nutrient reserves.

Bellamy, D. (1958). *Biochem. J.* **70**, 580–589. The structure and metabolic properties of tissue preparations from *Schistocerca gregaria* (Desert Locust).

Belzecka, K., Raczynska-Bojanowska, K. and Heller, J. (1959). *Acta Biochim. Pol.* **6**, 195–203. Studies on transamination in insects. I—Aspartate-α-ketoglutarate transaminase in *Celerio euphorbiae* L.

Blaschko, H., Colhoun, E. H. and Frontali, N. (1961). *J. Physiol.* **156**, 28. Occurrence of amine oxidase in an insect, *Periplaneta americana* L.

Brighenti, A. (1941). *R. C. Accad. Ital.* **2**, 915–918. Esperienze dirette a precisare i processi di formazione dell 'acido irico rei bachi da seta.

Brown, A. W. A. (1936). *J. exp. Biol.* **13**, 131–139. The excretion of ammonia and uric acid during the larval life of certain muscoid flies.

Brown, A. W. A. (1938a). *Biochem. J.* **32**, 895–902. The Nitrogen metabolism of an insect. I—Uric acid, allantoin and uricase.

Brown, A. W. A. (1938b). *Biochem. J.* **32**, 903–912. The Nitrogen metabolism of an insect. II—Ammonia and other metabolites.

Brown, A. W. A. and Farber, L. (1936). *Biochem. J.* **30**, 1107–1117. On the deaminating enzyme of Fleshfly larvae.

Buck, J. B. (1953). Physical properties and chemical composition of insect blood. *In* "Insect Physiology", (Ed. Roeder, K. D.)., pp. 147–190. John Wiley, New York.

Bückmann, D. (1959). *J. insect Physiol.* **3**, 159–189. Die Auslösung der Umfärbung durch das Hautungshormon bei *Cerula vinula* L. (Lepidoptera, Notodontidae).

Buys, K. S. (1924). *J. Morph.* **38**, 485–527. Adipose tissue in insects.

Cabib, E. and Leloir, L. F. (1958). *J. biol. Chem.* **231**, 259–275. The biosynthesis of trehalose phosphate.

Candy, D. J. and Kilby, B. A. (1959). *Nature, Lond.* **183**, 1594–5. Site and mode of trehalose biosynthesis in the locust.

Candy, D. J. and Kilby, B. A. (1961). *Biochem. J.* **78**, 531–536. The biosynthesis of trehalose in the locust fat body.

Carpenter, W. D. and Jaworski, E. G. (1962). *Biochim. biophys. Acta* **58**, 369–371. The presence of isocitric lyase activity in the southern armyworm, *Prodenia eridania* (Cramer).

Chadwick, L. E. (1947). *Biol. Bull., Woods Hole*, **93**, 229–239. The respiratory quotient of *Drosophila* in flight.

Chance, B. and Sacktor, B. (1958). *Arch. Biochem. Biophys.* **76**, 509–531. Respiratory metabolism of insect flight muscle. II. Kinetics of respiratory enzymes in flight muscle sarcosomes.

Chefurka, W. (1954). *Enzymologia* **17**, 73–89. Oxidative metabolism of carbohydrates in insects. I-Glycolysis in the Housefly, *Musca domestica* L.

Chefurka, W. (1955). *Biochim. biophys. Acta*, **17**, 295–296. The occurrence of a direct oxidative pathway of carbohydrate metabolism in the fly, *Musca domestica.*

Chefurka, W. (1957). *Enzymologia* **18**, 209–227. Oxidative metabolism of carbohydrates in insects. II—Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the Housefly, *Musca domestica* L.

Chefurka, W. (1958a). *Canad. J. Biochem. Physiol.* **36**, 83–102. Oxidative metabolism of carbohydrates in insects. III—Hexose monophosphate oxidative cycle in the Housefly, *Musca domestica* L.

Chefurka, W. (1958b). *Biochim. biophys. Acta*, **28**, 660–1. On the importance of α-glycerophosphate dehydrogenase in glycolysing insect muscle.

Chefurka, W. (1959). Glucose metabolism in insects. In "Biochemistry of Insects" (Symposium XII of the IVth Int. Congress Biochem. Ed. Levenbook, L.). Pergamon Press.

Clegg, J. S. and Evans, D. R. (1961). *J. exp. Biol.* **38**, 771–792. The physiology of blood trehalose and its function during flight in the Blowfly.

Clements, A. N. (1955). *J. exp. Biol.* **32**, 547–554. The source of energy for flight in Mosquitoes.

Clements, A. N. (1959). *J. exp. Biol.* **36**, 665–675. Studies on the metabolism of Locust fat body.

Cohen, A. J. and Smith, J. N. (1962). *Biochem. J.* **82**, 31. The metabolism of *p*-nitrobenzylchloride in Locusts.

Collin, G. (1933). *Biochem. J.* **27**, 1373–4. Fatty acids from the larval fat of the beetle *Pachymerus dactris* L.

Cromartie, R. I. T. (1959). *Ann. Rev. Entomol.* **4**, 59–76. Insect pigments.

Coupland, R. E. (1957). *J. exp. Biol.* **34**, 290–296. Observations on the normal histology and histochemistry of the fat body of the Locust (*Schistocerca gregaria*).

Davis, R. A. and Fraenkel, G. (1940). *J. exp. Biol.* **17**, 402–7. The oxygen consumption of flies during flight.

Day, M. F. (1951). *Aust. J. sci. Res. B4*, 64–74. Studies on the digestion of wool by insects. III—A comparison between the tracheation of the midgut of *Tineola* larvae and that of other insects.

Delbrück, A., Zebe, E. and Bücher, Th. (1959). *Biochem. Z.* **331**, 273–296. Über Verteilungsmuster von Enzymen des energie-liefernden Stoffwechsels im Flugmuskel, Sprungmuskel und Fettkörper von *Locusta migratoria* und ihre cytologische Zuordnung.

Desai, R. M. and Kilby, B. A. (1958a). *Arch. int. Physiol. Biochem.* **66**, 248–259. Some aspects of the nitrogen metabolism in the fat body of the larva of *Calliphora erythrocephala.*

Desai, R. M. and Kilby, B. A. (1958b). *Arch. int. Physiol. Bioch.* **66**, 282–286. Experiments on uric acid synthesis by insect fat body.

Diggle, W. M. and Gage, J. C. (1951). *Nature, Lond.* **168**, 998. Cholinesterase inhibition by Parathion *in vivo.*

Duchateau, G. and Florkin, M. (1959). *Arch. int. Physiol. Bioch.* **67**, 306–314. Sur la trehalosemie des insectes et sa signification.

Errara, M. (1949). *J. biol. Chemem.* **178**, 483–493. Liver glutaminases.

Fabre, J. H. (1856). *Ann. Sci. nat., Zool.* Ser. 4, **6**, 137–183. Étude sur l'instinct et les métamorphoses des Sphégiens.

Fairbairn, D. (1958). *Can. J. Zool.* **36**, 787–95. Trehalose and glucose in Helminths and other invertebrates.

Faulkner, P. (1956). *Biochem. J.* **64**, 403–436. The "Malic" enzyme in insect blood.

Faulkner, P. (1958). *Biochem. J.* **68**, 374–379. Polyol dehydrogenase of the Silkworm.

Fawzi, M., Osman, H. and Schmidt, G. H. (1961). *Biochem. Z.* **334**, 441–450. Analyse der Körperfette von imaginalen Wanderheuschrecken der Art *Locusta migratoria migratorioides* L. (Orth.).

Fenwick, M. L. (1958a). *Biochem. J.* **70**, 373–381. The production of an esterase inhibitor from Schradan by the fat body of the Desert Locust.

Fenwick, M. L. (1958b). *Nature, Lond.* **182**, 607. Intracellular particles of Locust fat body.

Florkin, M. (1949). "Biochemical Evolution", 157 pp., Academic Press, New York.

Florkin, M. and Duchateau, G. (1943). *Arch. int. Physiol. Biochem.* **53**, 267–307. Les formes du système enzymatique de l'urocolyse et l'évolution du catabolisme purique chez les animaux.

Frerejacque, M. M. (1941). *C. R. Acad. Sci., Paris* **213**, 88–90. Trehalose et trehalase.

Friedman, S. (1960a). *Arch. Biochem. Biophys.* **88**, 339–343. Occurrence of trehalose-6-phosphatase in *Phormia regina* Meig.

Friedman, S. (1960b). *Arch. Biochem. Biophys.* **87**, 252–258. The purification and properties of trehalase isolated from *Phormia regina* Meig.

Friedman, S. (1961). *Arch. Biochem. Biophys.* **93**, 550–554. Inhibition of trehalase activity in the hemolymph of *Phormia regina* Meig.

Fukuda, T. (1957). *J. Biochem., Tokyo* **44**, 505–510. The conversion of pyruvic acid to alanine in the silkworm larva.

Gardiner, J. E. and Kilby, B. A. (1952). *Biochem. J.* **51**, 78–85. Biochemistry of organic phosphorus Insecticides. I—The mammalian metabolism of *bis* (dimethylamino) phosphonous anhydride (Schradan).

Gary, N. E., Berger, R. S. and Toung, R. G. (1959). *Ann. ent. Soc. Amer.* **52**, 570–573. Biology and dehydrogenase activities of the fat body of late instar American cockroaches (*Periplaneta americana*).

George, J. C. and Eapen, J. (1959a). *Nature, Lond.* **183**, 268. Histochemical demonstration of lipase and alkaline phosphatase activity in the fat body of the Desert Locust.

George, J. C. and Eapen, J. (1959b). *J. cell. comp. Physiol.* **54**, 293–295. Lipase activity in the fat body of the Desert Locust, *Schistocerca gregaria.*

George, J. C. and Hegdekar, B. M. (1961). *J. histochem. cytochem.* **9**, 157–160. Histochemical demonstration of succinic dehydrogenase in the fat body of the Desert Locust and some grasshoppers.

Gilmour, D. (1961). "The Biochemistry of Insects", 343 pp., Academic Press, New York.

Giral, F. (1946). *J. biol. Chem.* **162**, 61–63. Fats of insects. V—*Sphenarium purpurascens* Carpentier.

Giral, J., Giral, F. and Giral, M. L. (1946). *J. biol. Chem.* **162**, 55–59. Composition of the fat of *Melanoplus atlantis* Riley.

Glassman, E. and Mitchell, H. K. (1959). *Genetics* **44**, 153–162. Mutants of *Drosophila melanogaster* deficient in xanthine dehydrogenase.

Goodwin, T. W. (1952). *Biol. Rev.* **27**, 439–460. The biochemistry of insect pigmentation.

Goodwin, T. W. and Srisukh, S. (1949). *Biochem. J.* **45**, 263–268. The carotenoids of the integument of two locust species (*Locusta migratoria migratorioides* R & F and *Schistocerca gregaria* Forsk.).

Gregg, C. T., Heisler, C. R. and Remmert, L. F. (1960). *Biochim. Biophys. Acta* **45**, 561–570. Oxidative phosphorylation and respiratory control in housefly mitochondria.

Grindley, D. N. (1952). *J. exp. Biol.* **29**, 440–444. Composition of the body fat of small green chironomids.

Hadorn, E. and Schwinck, I. (1956). *Nature, Lond.* **177**, 940–1. A mutant of *Drosophila* without *iso*-xanthopterin which is non-autonomous for the red eye pigments.

Handel, E. van and Lum, P. T. M. (1961). *Science* **134**, 1979–80. Sex as regulator of triglyceride metabolism in the Mosquito.

Hayashi, Y. (1962). *Biochim. Biophys. Acta* **58**, 351–2. Some properties of the xanthine dehydrogenase of the silkworm, *Bombyx mori* L.

Haydak, M. H. (1953). *Ann. ent. Soc. Amer.* **46**, 547–560. Influence of the protein level of the diet on the longevity of cockroaches.

Hearfield, D. A. H. and Kilby, B. A. (1958). *Nature, Lond.* **181**, 546–7. Enzymes of the tricarboxylic acid cycle and cytochrome oxidase in the fat body of the Desert Locust.

Heller, J. and Jezewska, M. M. (1959). *Bull. Acad. pol. Sci.* Cl. II, **7**, 1–4. The synthesis of uric acid by the Chinese Tussur Moth (*Antheraea pernyi*).

Hemmingsen, A. M. (1924). *Skand. Arch. Physiol.* **45**, 204–210. The blood sugar of some invertebrates.

Henry, S. M. and Block, R. J. (1960). *Contr. Boyce Thompson Inst.* **20**, 317–329. The conversion of inorganic sulphate to organic sulphur compounds in cockroaches. The role of intracellular symbionts.

Henry, S. M. and Block, R. J. (1962). *Fed. Proc.* **21**, 9. Amino acid synthesis, a rumen-like effect of the intracellular symbionts of the German cockroach.

Hilditch, T. P. (1956). "The Chemical Constitution of Natural Fats", 3rd ed., 664 pp., Chapman and Hall, London.

Hinton, H. E. (1958). *J. ins. Physiol.* **2**, 249–260. On the nature and metamorphosis of the colour pattern of *Thaumalea* (Diptera, Thaumaleidae).

Hird, F. J. R. and Rowsell, E. V. (1950). *Nature, Lond.* **166**, 517–518. Additional transaminations by insoluble particle preparations of rat liver.

Hollande, A. C. (1913). *Arch. Zool.* **51**, 53–58. Coloration vitale du corps adipeux d'un insecte phytophage par une anthocyane absorbée avec la nourriture.

Hollande, A. C. (1914). *Arch. Zool.* **53**, 559–578. Formations endogènes des cristalloides albuminoides et des urates des cellules adipeuses des chenilles de *Vanessa io* et *Vanessa urticae*.

Hoskins, D. D., Cheldelin, V. H. and Newburgh, R. W. (1956). *J. gen. Physiol.* **39**, 705–713. Oxidative enzyme systems of the Honey bee, *Apis mellifera* L.

Howden, G. F. and Kilby, B. A. (1956). *Chem. & Ind.* (*Rev.*) 1453–4. Trehalose and trehalase in the Locust.

Howden, G. F. and Kilby, B. A. (1957). Unpublished results.

Howden, G. F. and Kilby, B. A. (1961). *J. insect Physiol.* **6**, 85–95. Biochemical studies on insect haemolymph. II—The nature of the reducing material present.

Hudson, A. (1958). *J. ins. Physiol.* **1**, 293–304. The effect of flight on the taste threshold and carbohydrate utilisation of *Phormia reginia* Meigen.

Hudson, B. W., Bartel, A. H. and Craig, R. (1959). *J. ins. Physiol.* **3**, 63–73. Pteridines in the milkweed bug, *Oncopeltus fasciatus* Dallas. II. Quantitative determination of pteridine content of tissues during growth.

Irzykiewicz, H. (1955). *Aust. J. biol. Sci.* **8**, 369–377. Xanthine oxidase of the clothes moth, *Tineola bisselliella*, and some other insects.

Kalf, G. F. and Rieder, S. V. (1958). *J. biol. Chem.* **230**, 691–698. The purification and properties of trehalase.

Keilin, D. and Wang, Y. L. (1946). *Biochem. J.* **40**, 855–866. Haemoglobin of *Gastrophilus* larvae. Purification and properties.

Keller, H. (1950). *Z. Naturforschg.* **5B** 269–273. Die Kultur der intrazellularen Symbioten von *Periplaneta orientalis*.

Kerly, M. and Leaback, D. H. (1957). *Biochem. J.* **67**, 245–250. The characteristics of hexokinase from *Locusta migratoria* muscle.

Kikkawa, H. (1953). *Advances in Genetics* **5**, 107–145. Biochemical genetics in *Bombyx mori*.

Kilby, B. A. and Neville, E. (1957). *J. exp. Biol.* **34**, 276–289. Amino acid metabolism in Locust tissues.

Kok, G. C. and Walop, J. N. (1954). *Biochim. biophys. Acta* **13**, 510–515. Conversion of O,O-diethyl-O-*p*-nitrophenyl thiophosphate (parathion) into an acetylcholinesterase inhibitor by the insect fat body.

Kuwana, Z. (1937). *Jap. J. Zool.* **7**, 273–303. Reducing power of the body fluid of the Silkworm.

Lennox, F. G. (1941). *Aust. Council Sci. Ind. Res. Pamphlet* **109**, 9–35. The physiology and toxicology of blowflies. VIII—Rate of ammonia production by larvae of *Lucilia cuprina* and its distribution in this insect.

Levenbook, L. (1950). *Biochem. J.* **47**, 336–346. The composition of Horse Bot Fly (*Gastrophilus intestinalis*) larva blood.

Levenbook, L. (1951). *J. exp. Biol.* **28**, 173–180. The variation in fat and glycogen content of the Bot Fly (*Gastrophilus intestinalis*) larva tracheal organ during development.

Levenbook, L. (1961). *Arch. Biochem. Biophys.* **92**, 114–121. Organic acids in insects. II—Tricarboxylic acid cycle and related enzymes in the larva of the Southern Armyworm, *Prodenia eridania*.

Levenbook, L. and Hollis, V. W. (1961). *J. ins. Physiol.* **6**, 52–61. Organic acids in insects. I—Citric acid.

Liefert, H. (1935). *Zool. Jb. Abt. Allg. Zool. Physiol.* **55**, 171–190. Die Bildungsmöglichkeit von Harnsäure und Allantoin im überlebenden Gewebe der Raupen und Puppen von *Antheraea pernyi*.

Liza, J. D. and Ludwig, D. (1959). *Ann. ent. Soc. Amer.* **52**, 548–551. Uricase, guanase and xanthine oxidase from the fat body of the cockroach, *Leucophaea maderae*.

Ludwig, D. (1954). *Physiol. Zool.* **27**, 325–334. Changes in distribution of nitrogen in blood of Japanese beetle, *Popillia japonica* Newman, during growth and metamorphosis.

McAllen, J. W. (1958). Ph.D. thesis. Cited by Bheemeswar, B. in "Biochemistry of Insects" (1959), 78–89, Pergamon Press, London.

McEnroe, W. D. and Forgash, A. J. (1957). *Ann. ent. Soc. Amer.* **50**, 429–431. The *in vivo* incorporation of ^{14}C formate in the ureide groups of uric acid by *Periplaneta americana* L.

McEnroe, W. D. and Forgash, A. J. (1958). *Ann. ent. Soc. Amer.* **51**, 126–129. Formate metabolism in the American cockroach, *Periplaneta americana* L.

McGinnis, A. J., Cheldelin, V. H. and Newburgh, R. W. (1956). *Arch Biochem. Biophys.* **63**, 427–436. Enzyme studies of various stages of the blowfly, *Phormia regina* (Meig).

Manunta, C. (1948). *R. C. Accad. Lincei* Ser. 8A **4** (2), 211–214. (Not seen; *Biol. Abst.* **24** (1950) No 8723). Sul metabolismo dell' azoto in *Bombyx mori.* IV—Ricerca degli enzimi allantoicasi ed allantoinasi nei bachi da seta durante lo iviluppo larvali.

Metcalf, R. L. (1943). *Ann. ent. Soc. Amer.* **36**, 37–40. The isolation of a red-fluorescent pigment, lampyrine, from the *Lampyridae.*

Metcalf, R. L. (1945). *Ann. ent. Soc. Amer.* **38**, 397–402. A study of the metabolism of chlorophyll in the Squash bug, *Anasa tristis* DeGeer.

Meyer, G. F. and Frank, W. (1957). *Z. Zellforschung.* **47**, 29–42. Elektronenmikroskopische Studien zur intrazellulären Symbiose verschiedener Insekten. I—Untersuchungen des Fettkörpers und der symbiontischen Bakterien der Küchenschabe (*Blatta orientalis* L).

Myers, C. M. and Smith, J. N. (1954). *Biochem. J.* **56**, 498–503. Glucoside formation from phenols in Locusts.

Nakamura, M., Pichette, S., Broitman, S., Bezman, A. L., Zamcheck, N. and Vitale, J. J. (1959). *J. biol. Chem.* **234**, 206–210. Studies on the inhibitory effect of intestinal mucosa on succinoxidase activity.

Nelson, W. A. (1958). *Nature, Lond.* **182**, 115. Purine excretion by the sheep Ked, *Melophagus ovinus* (L).

Newburgh, R. W. and Cheldelin, V. H. (1955). *J. biol. Chem.* **214**, 37–45. Oxidation of carbohydrates by the pea aphid, *Macrosiphum pisi* (Kltb).

Oertel, E. (1930). *J. Morph.* **50**, 295–339. Metamorphosis in the Honeybee.

Pearse, A. G. E. (1959). Quoted in Clements, A. N. (1959).

Pearse, A. G. E. and Scarpelli, D. G. (1958). *Nature, Lond.* **181**, 702–3. Cytochemical localization of succinic dehydrogenase in mitochondria from *Periplaneta americana.*

Pepper, J. H. and Hastings, E. (1943). *Montana Agr. Sta. Tech. Bull.* 413. (Not seen.). Biochemical studies on the sugarbeet webworm (*Loxostege sticticalis* L.) with special reference to the fatty acids and their relation to diapause and sterility.

Pierre, L. L. (1962). *Nature, Lond.* **193**, 904–5. Synthesis of Ascorbic acid by the normal fat body of the cockroach, *Leucophaea maderae* (F) and by its symbionts.

Poisson, R. and Razet, P. (1953). *C. R. Acad. Sci., Paris* **237**, 1362–3. Recherches sur la degradation de l'acide urique chez les Hemipteres aquatique.

Popjak, G. and Tietz, A. (1954). *Biochem. J.* **56**, 46–54. Biosynthesis of fatty acids by slices and cell-free suspensions of mammary gland.

Porter, J. W., Wakil, S. J., Tietz, A., Jacob, M. I. and Gibson, D. M. (1957). *Biochim biophys. Acta* **25**, 35–41. Studies on the mechanism of fatty acid synthesis. II–Cofactor requirement of the soluble pigeon liver system.

Razet, P. (1953). *C. R. Acad. Sci., Paris* **236**, 1304–6. Recherches sur la localisation des enzymes uricolytique chez les Insectes.

Razet, P. (1954). *C. R. Acad. Sci., Paris* **239**, 905–907. Sur l'elimination d'acide allantoique par quelque Insectes Lepidopteres.

Razet, P. (1956). *C. R. Acad. Sci., Paris* **243**, 185–7. Sur l'elimination simultanee d'acide urique et d'acide allantoique chez les Insectes.

Rizki, M. T. M. (1961). *J. Biophys. Biochem. Cyt.* **9**, 567–572. Intracellular localization of kynurenine in the fat body of *Drosophila.*

Rocco, M. (1936). *C. R. Acad. Sci., Paris* **202**, 1947–8. Presence de l'allantoïnase chez des Insectes.

Rockstein, M. (1950). *Bull. Brooklyn ent. Soc.* **45**, 74–81. Glycogen metabolism in insects.

Ross, D. J. (1959). *Physiol. Zool.* **32**, 239–245. Changes in activities of uricase and xanthine oxidase during life-cycle of Japanese beetle (*Popillia japonica*).

Rousell, P. G. (1956). *Trans. N.Y. Acad. Sci.* Ser. 2, **19**, 17–18. Effects of various factors on the synthesis of ascorbic acid by the American cockroach, *Periplaneta americana* L.

Sacktor, B. and Bodenstein, D. (1952). *J. cell. comp. Physiol.* **40**, 157–161. Cytochrome *c* oxidase activity of various tissues of the American cockroach, *Periplaneta americana.*

Sacktor, B. and Cochran, D. G. (1958). *Arch. Biochem. Biophys.* **74**, 266–276. The respiratory mechanism of flight muscle. I—Manometric studies of oxidation and concomitant phosphorylation with sarcosomes.

Sacktor, B. and Thomas, G. M. (1955). *J. cell. comp. Physiol.* **45**, 241–245. Succino-cytochrome *c* reductase activity of tissues of the American cockroach, *Periplaneta americana* (L).

Saito, S. (1960). *J. Biochem., Tokyo* **48**, 101–9. Trehalase of the silkworm, *Bombyx mori.* Purification and properties of the enzyme.

Salt, R. W. (1958). *Nature, Lond.* **181**, 1281. Role of glycerol in producing abnormally low supercooling and freezing points in an insect, *Bracon cephi* (Gahan).

Shappirio, D. G. and Williams, C. M. (1957a). *Proc. roy. Soc.* B **147**, 218–232. The cytochrome system of the Cecropia silkworm. I—Spectroscopic studies of individual tissues.

Shappirio, D. G. and Williams, C. M. (1957b). *Proc. roy. Soc.* B **147**, 233–246. The cytochrome system of the Cecropia silkworm. II—Spectroscopic studies of oxidative enzyme systems in the wing epithelium.

Shigematsu, H. (1956). *J seric. Sci., Tokyo* **25**, 115–121. On the glycolytic and oxidative action by the enzymes in the fat body of the silkworm, *Bombyx mori* L.

Shigematsu, H. (1958a). *Nature, Lond.* **182**, 880–882. Synthesis of blood proteins by the fat body in the Silkworm, *Bombyx mori* L.

Shigematsu, H. (1958b). *Annot. zool. jap.* **31**, 6–12. Hexokinase in the fat body of the silkworm, *Bombyx mori* L.

Shigematsu, H. (1960a). *J. seric. Sci., Tokyo* **29**, 22–27. Utilisation of glucose by fat body of the silkworm *in vitro.*

Shigematsu, H. (1960b). *Bull. seric. Exp. Sta. Japan* **16**, 141–170. Protein metabolism in the fat body of the silkworm, *Bombyx mori* L.

Smith, J. N. and Turbert, H. B. (1961). *Nature, Lond.* **189**, 600. Enzymic glucoside synthesis in Locusts.

Spencer, E. Y. and O'Brien, R. D. (1957). *Annu. Rev. Entomol.* **2**, 261–278. Chemistry and mode of action of organophosphorus insecticides.

Szarkowska, L. and Porembska, Z. (1959). *Acta Biochim. Pol.* **6**, 273–276. Arginase in *Celerio euphorbiae.*

Tietz, A. (1961). *J. Lipid Res.* **2**, 182–187. Fat synthesis in cell-free preparations of the locust fat body.

Timon-David, J. (1928). *C. R. Acad. Sci., Paris* **186**, 104–106. Contributions à la connaissance des graisses d'Insectes: le beurre de Pucerons du Térébinthe.

Treherne, J. E. (1958a). *J. exp. Biol.* **35**, 297–306. The absorption of glucose from the alimentary canal of the locust, *Schistocerca gregaria* Forsk.

Treherne, J. E. (1958b). *J. exp. Biol.* **35**, 611–625. The absorption and metabolism of some sugars in the locust, *Schistocerca gregaria* Forsk.

Trivelloni, J. C. (1960). *Arch. Biochem. Biophys.* **89**, 149–150. Biosynthesis of glucosides and glycogen in the Locust.

Urich, K. (1961). *Ergebn. Biol.* **24**, 155–190. Mitteldarmdrüsen und Insektenfettkörper als Zentralorgane des Stoffwechsels.

Ursprung, H. (1961). *Experientia* **17**, 232–3. Xanthindehydrogenase beim Wildtyp und bei den Mutaten *white* und *brown* von *Drosophila melanogaster*.

Ursprung, H. and Hadorn, E. (1961). *Experientia*, **17**, 230–1. Xanthindehydrogenase in Organen von *Drosophila melanogaster*.

Wakil, S. J. and Ganguly, P. (1959). *J. Amer. chem. Soc.* **81**, 2597–8. On the mechanism of fatty acid synthesis.

Wiener, H. (1902). *Ergebn. Physiol.* **1**, 555–650. Die Harnsäure.

Weis-Fogh, T. (1952). *Phil. Trans.* Ser. B **237**, 1–36. Fat combustion and metabolic rate of flying locusts (*Schistocerca gregaria* Forskal).

Wigglesworth, V. B. (1931). *J. exp. Biol.* **8**, 411–427. The physiology of excretion in a blood-sucking insect, *Rhodnius prolixus* (Hemiptera, Reduviidae). I—Composition of the urine.

Wigglesworth, V. B. (1942). *J. exp. Biol.* **19**, 56–77. The storage of protein, fat, glycogen and uric acid in the fat body and other tissues of Mosquito larvae.

Wigglesworth, V. B. (1943). *Proc. roy. Soc.* **B 131**, 313–339. The fate of haemoglobin in *Rhodnius prolixus* (Hemiptera) and other blood sucking arthropods.

Wigglesworth, V. B. (1949). *J. exp. Biol.* **26**, 150–163. The utilisation of reserve substances in *Drosophila* during flight.

Wigglesworth, V. B. (1953). "The principles of Insect Physiology", pp. 546, Methuen, London.

Wigglesworth, V. B. (1958). *Quart. J. micr. Sci.* **99**, 441–450. The distribution of esterase in the nervous system and other tissues of the insect *Rhodnius prolixus*.

Wigglesworth, V. B. (1959). "The Control of Growth and Form: A Study of the Epidermal Cell in an Insect", pp. 140, Cornell University Press, N.Y.

Williams, C. M. (1947). *Anat. Rec.* **99**, 591. The cytochrome system in relation to diapause and development in the Cecropia silkworm.

Williams, C. M., Barnes, L. A. and Sawyer, W. H. (1943). *Biol. Bull., Woods Hole* **84**, 263–272. The utilisation of glycogen by flies during flight and some aspects of the physiological ageing of *Drosophila*.

Wren, J. J. and Mitchell, H. K. (1959). *J. biol. Chem.* **234**, 2823–8. Extraction methods and an investigation of *Drosophila* lipids.

Wyatt, G. R. and Kalf, G. F. (1956). *Fed. Proc.* (abstract 1269), **15**, 388. Trehalose in insects.

Wyatt, G. R. and Kalf, G. F. (1957). *J. gen. Physiol.* **40**, 833–847. The chemistry of insect hemolymph: II—Trehalose and other carbohydrates.

Young, R. G. (1958a). *J. econ. Ent.* **51**, 867–869. Dehydrogenase systems from fat body of susceptible and Chlordane resistant *Blattella germanica* (L).

Young, R. G. (1958b). *Int. Congr. Ent. Montreal* (1956) **2**, 369–370. Aldolase and malic dehydrogenase in the fat body of *Periplaneta americana.*

Young, R. G. (1959). *Ann. ent. Soc. Amer.* **52**, 567–570. Oxidative metabolism of insect fat body.

Zebe, E. (1958). *Int. Congr. Ent. Montreal* (1956), **2**, 371–376. Studies on Glycolytic enzymes in insect muscle.

Zebe, E. (1959). *Verh. d.dtsch. Zool in Münster/Westf.*, 309–314. Die Verteilung von Enzymen des Fettstoffwechsels im Heuschreckenkörper.

Zebe. E. (1960). *Biochem. Z.* **332**, 328–332. Condensing enzyme und β-Keto-acyl-thiolase in verschiedenen Muskeln.

Zebe, E. and McShan, W. H. (1959a). *Biochim. Biophys. Acta* **31**, 513–518. Incorporation of (^{14}C)-acetate into long chain fatty acids by the fat body of *Prodenia eridania* (Lep).

Zebe, E. and McShan, W. H. (1959b). *J. cell. comp. Physiol.* **53**, 21–29. Trehalase in the thoracic muscles of the Woodroach, *Leucophaea maderae.*

Ziegler, I. (1961). *Advanc. Genet.* **10**, 349–403. Genetic aspects of ommochrome and pterin pigments.

The Properties of Insect Axons

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I. INTRODUCTION

Remarkable progress has been achieved in neurophysiology since the establishment about twenty years ago of the micro-electrode technique, which has made it possible to record absolute values for membrane resting and action potentials from a wide variety of tissues. This development has greatly stimulated studies in the field of insect neurophysiology which has long been of great interest for several reasons.

Since insects constitute a large part of the animal kingdom, a wide variety of morphological and physiological variations can be found. For example, some insects are aquatic while others can tolerate dry conditions.

This difference leads one to expect different compositions of hemolymph, which may reflect different neurophysiological properties. Another example of physiological variation is found in phytophagous insects in which the ionic composition of hemolymph is quite different from that of others. This again raises the question whether the nervous system of such phytophagous insects is functionally peculiar. The morphological and physiological variations provide an excellent opportunity to study comparative neurophysiology.

The second reason comes from applied entomology. Since the development of organic insecticides after the Second World War, it has been agreed that knowing the normal physiological properties of insect nerves is prerequisite to elucidating the mode of action of various insecticides, most of which are neurotoxins.

There have recently appeared several reviews of the physiology of insect nerve and muscle. Nervous activity was reviewed by Roeder (1953a, b, 1958), Prosser (1946, 1950b, 1954), Bullock (1947), Wiersma (1953), Welsh and Schallek (1946), and Ten Cate (1931); sensory physiology by Frings and Frings (1958), Boistel (1960), Prosser (1954), Wulff (1956), Hodgson (1958), and Dethier (1953a–c); muscle physiology including flight by Hoyle (1957), Pringle (1957), Boettiger (1957, 1960), Wiersma (1952), Prosser (1950a), and Chadwick (1953a–c); behavior by Lehrman (1956), Baerends (1959) and Schneirla (1953a–c); and the properties of insect axons by Boistel (1960) and Narahashi (1960). In the present article, various physiological properties of insect nerves are described and discussed. Some morphological aspects are also included, because knowledge of morphology and histology will no doubt be helpful in understanding physiological properties. Attention is especially focused on the nature of the membrane potential, the mechanism of excitation, ionic fluxes across the axon membrane, the effect of ionic composition of the hemolymph on electrical excitability of nerve, and the effects of insecticides on electrical excitability. It is not the author's intention to cover evenly the whole field concerned.

II. Structure and Organization of the Nervous System

There are at least three aspects of morphology and histology of insect nerve which should be carefully examined to interpret physiological experiments. (1) Organization of the giant fiber system to which we apply micro-electrode techniques. (2) Fine structure of the axon membrane which is undoubtedly the important site of excitation. (3) Histology and

biochemistry of the perineural sheath, which may act as a diffusion barrier for ions. Only general outline will be supplied here. Interested readers may refer to articles by Edwards (1960) and Smith and Treherne (1963) concerning fine structure.

A. ORGANIZATION OF THE GIANT FIBER SYSTEM

1. *Cockroach nerve cord*

The giant axons in the nerve cord of the cockroach are widely used for physiological experiments. This is because (1) the diameter of the axons is large enough for micro-electrode impalement; (2) even with external electrodes the action potentials can easily be distinguished from those of smaller axons; and (3) this system provides us with an excellent opportunity to study synaptic transmission. The system was first used for physiological work some 25 years ago (Pumphrey and Rawdon-Smith, 1937). Since then it has received much attention both from physiologists and histologists, and the organization is now well established (Roeder, 1948a; Hess, 1958a).

In each connective, there are five to six giant axons whose diameters range from 20 to 60 μ. The largest three are located in the ventral part of the connective, while the others form a dorsal group. Besides these giant axons, there are 10 to 12 medium-sized axons having diameters of 5–20 μ. The giant axons pass through the abdominal ganglia, and there appear to be no septa or breaks at any point in the abdominal nerve cord. However, the axons become thinner as they pass through the ganglia. Thus in the abdominal ganglia, the dorsally located giants are about 20 μ which is less than half their diameter in the connectives. In the last (sixth) abdominal ganglion, the giant axons synapse with the cervical nerve. The cell bodies of the giant axons in the last abdominal ganglion are arranged in clusters of from three to six. No afferent fibers from the cercus enter the abdominal nerve cord without synapsing in the last abdominal ganglion. Some of the giant axons end in the meta-thoracic ganglion and make synaptic contacts with motoneurones which innervate the muscles of the legs. Some small axons apparently ascend to the brain without interruption. Evidence is obtained by observation of degeneration that some giant axons have a multi-cellular origin with their cell bodies perhaps located in every abdominal ganglion. Most of the largest axons serve as a pathway for ascending impulses, while some probably transmit descending impulses. It is apparent that a pathway from the cercus to the leg muscle via the cercal nerve, the giant axons and the motoneurones establishes an evasion reflex.

2. *Grasshopper nerve cord*

The giant fiber system of the grasshopper, *Locusta migratoria*, was observed by Cook (1951). Four large fibers, originating in the last (fifth) abdominal ganglion, pass through all intervening ganglia without interruption to the meta-thoracic ganglion. These four fibers are arranged in two groups: a single median fiber (13 μ) and a peripheral latero-dorsal group of three fibers (two of 12 μ and one of 8·5 μ). They receive synaptic contacts from the cercal nerve fibers in the last abdominal ganglion, and connect synaptically with motoneurones in the meta-thoracic ganglion. As in the cockroach, an evasion reflex is established by the pathway from the cercus to the leg muscles via the cercal nerve, the giant axons and the motoneurones.

3. *The nerve cord of the dragonfly nymph*

There are six to seven giant axons of 12–16 μ diameter in the abdominal nerve cord of the dragonfly nymph, *Anax imperator* (Fielden, 1960; Hughes, 1953). In the last (seventh) abdominal ganglion, the afferent nerve fibers from the tactile endings on the paraprocts make synaptic contacts with these giant axons, and the latter run through the abdominal nerve cord and connect synaptically with the efferent nerve fibers in the thoracic and abdominal ganglia. Again an evasion reflex pathway is established.

B. HISTOLOGY OF THE NERVOUS SYSTEM

The nerve cord is surrounded by a sheath in which two layers can be distinguished, an outer non-cellular and an underlying cellular one. The outer non-cellular layer is called the neural lamella (Schneider, 1902; Scharrer, 1939; Hoyle, 1952; Hess, 1958b; Imms, 1957) or perilemma (Wigglesworth, 1956), and the underlying cellular layer is called perineurium (Schneider, 1902; Scharrer, 1939; Wigglesworth, 1956; Smith and Treherne, 1963), epineurium (Imms, 1957) perilemma (Hoyle, 1952; Hess, 1958b), Schwann cell layer (Hess, 1958c) or lemnoblast (Edwards, 1957, 1959). In order to avoid confusion, only the terms neural lamella and perineurium will be used here.

The neural lamella contains a collagen-like protein (Ashhurst, 1959; Baccetti, 1957; Richards, 1944; Rudall, 1955; Smith and Wigglesworth, 1959; Wigglesworth, 1959a, 1960). It is probably the perineurium that regulates transfer of ions and acts as a diffusion barrier (Ashhurst, 1959; Twarog and Roeder, 1956; Wigglesworth, 1959a, 1960).

The plasma membrane of the cellular perineurium may invaginate at

several points and form mesaxons (Edwards, 1957, 1959; Edwards and Challice, 1960; Edwards *et al.*, 1958a, b; Hess, 1958c). The individual axon is suspended within a single turn of the mesaxon or within several loose mesaxonal turns. Thus the nerve fibers are "tunicated" rather than "myelinated" (Edwards, 1959; Edwards and Challice, 1960; Edwards *et al.*, 1958b). It seems likely that the mesaxonal turns surrounding the individual axon corresponds to a "delicate sheath-like structure" observed by Twarog and Roeder (1956) and a "concentric structure which is regarded as a primitive type of myelin sheath" observed by Wigglesworth (1959b). Each axon is enclosed by a thin axolemma and contains axoplasmic mitochondria. It is generally believed that the axolemma corresponds to the "nerve membrane", the name which is usually used by physiologists.

III. Membrane Potential and Electrical Excitability

It was not until the discovery of the overshoot of the membrane potential during excitation that the mechanism of action potential production could be interpreted on a physico-chemical basis. Until that time it had long been believed (Bernstein, 1912) that the membrane potential, inside negative, temporarily vanished during an action potential. The resting potential was measured as an injury potential or a potassium potential by means of external electrodes in such a way that the short circuit caused by the axoplasm and the external fluid which adhered to the nerve fibers made the recorded resting potential smaller than the absolute value. However, some 20 years ago, two groups of physiologists attempted to record the absolute values of the resting and action potentials (Curtis and Cole, 1940; Hodgkin and Huxley, 1939). The measurement was made by introducing one electrode longitudinally into the axoplasm of a squid giant axon having a diameter as much as 500 μ or more, while another electrode was dipped into the external solution. Surprisingly, the action potential thus recorded showed an overshoot which was not expected from the classical membrane theory of Bernstein (1912).

However, this method of recording the membrane potential required axons large enough to permit a longitudinal electrode to be inserted. Shortly afterwards another method was successfully attempted (Graham and Gerard, 1946; Ling and Gerard, 1949a–c; Ling and Woodbury, 1949). This method depended on inserting a fine glass capillary filled with salt solution transversely into a muscle fiber, and was improved to permit recording of the action potential as well as of the resting potential (Nastuk and Hodgkin, 1950). A quite different method of measuring the

absolute values of the potentials from myelinated nerve fibers by external electrodes was developed by Huxley and Stämpfli (1951a).

The development of the capillary micro-electrode made it possible to record membrane resting and action potentials from a wide variety of excitable tissues. It is only natural that this technique has also been used with insect nerve. The first attempt was made by Boistel and Coraboeuf (1954) in France, using cockroach giant axons. Some time later a similar attempt was made in Tokyo by the author in collaboration with Dr. T. Yamasaki (Yamasaki and Narahashi, 1957c). These studies were extended, and the normal properties of insect axons were explored as well as were the effects of various insecticides upon them. The latter problem has also been studied by means of the classical external electrodes by several investigators including the author. Hoyle (1953) and Hagiwara (1953) applied the micro-electrode technique to the locust muscle fibers. An attempt was also made to record the membrane resting and action potentials from the nerve cells of cicada by Higawara and Watanabe (1956). Electrical responses from the retinas of insects were also subjected to micro-electrode analysis (Naka and Kuwabara, 1959; see also Wulff, 1956). Nowadays, the intracellular micro-electrode technique is widely used in nerve and muscle of insects.

A. METHODS

It is not the principal aim to introduce the detailed method of recording membrane potential. However, it was felt that brief explanation would be helpful in understanding the problems concerned.

1. *Electrodes*

External electrodes, of whatever type, are so familiar that they need not be discussed here.

Micro-electrodes are mechanically pulled from glass tubes, usually of hard glass, of about 0·8–1·5 mm outer diameter. The tip of the micro-electrode should be as small as 1 μ or less in diameter. They are then filled with 3M KCl solution. It is possible to fill them by simple boiling in KCl, but it is preferable to use the "alcohol method" developed by Tasaki *et al.* (1954) in order to avoid damage to the tip. This method depends on boiling the empty micro-electrodes in methanol under reduced pressure at room temperature, and transferring them to distilled water followed by further transfer to 3M KCl. The electrodes are kept in distilled water for about 1 h during which methanol in the capillaries is replaced with water by diffusion, and then should be kept in the KCl solution for at least 1 day to enable water to be replaced by the KCl solution. The resistance of the

KCl-filled micro-electrodes should be in the range of 5–20 MΩ for use in cockroach giant axons. The optimum resistance and diameter are, of course, different depending on the material used.

2. *Nerve chamber*

Since most insect nerve preparations are relatively small, a nerve chamber should be devised depending on the size of the preparation, the purpose of study and the methods of recording. A difficulty usually encountered in addition to the small size is the inability of most insect nerves to tolerate mineral oil such as liquid paraffin. This causes great trouble when the recording is made by means of external electrodes, because it is

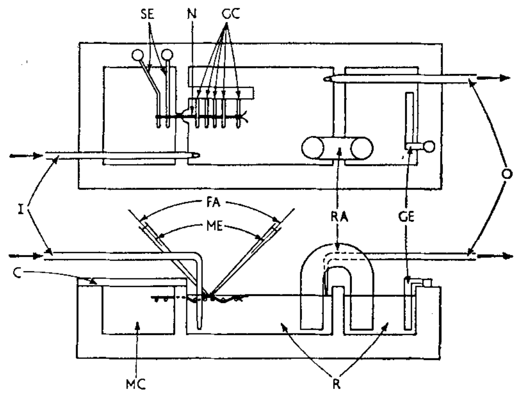

FIG. 1. Nerve chamber for micro-electrode experiment with the cockroach giant axons. C, cover; FA, fine Ag-AgCl wire; GC, glass capillaries to support the nerve preparation; GE, ground electrode (Ag-AgCl); I, inlet of bathing fluid; MC, moist chamber; ME, micro-electrode; N, nerve cord preparation; O, outlet of bathing fluid; R, Ringer; RA, Ringer-agar bridge; SE, stimulating electrode (Ag).

needed in order to reduce short circuiting as much as possible. When the excised preparation is used, one may devise a moist chamber or dip the whole preparation into a physiological medium. In the latter case, several compartments may be used so that each acts as an electrode. However, the difficulty is greatest when the recording is made *in situ*. In this case, one requires a suitable moist chamber, or physiological solution must be applied from time to time to prevent drying. The moist chamber has proved to be useful when the humidity is relatively high, as it is in Japan, but it seems more difficult to apply under dry conditions.

When intracellular micro-electrodes are used, the situation is much simpler as far as drying the preparation is concerned, because with this method one can keep the whole preparation in the physiological solution. Figure 1 shows an example of a nerve chamber used for this purpose by the author. For impalement by micro-electrodes, it is absolutely necessary

to hold the preparation tightly, especially when two micro-electrodes are used and the external medium is changed while keeping the micro-electrodes inserted. The moist chamber shown in Fig. 1 may be removed, soaking the whole of the preparation in the physiological solution, but in this case it is advisable that external stimuli be applied by a pair of wire electrodes insulated except for a small patch with which the preparation is in contact.

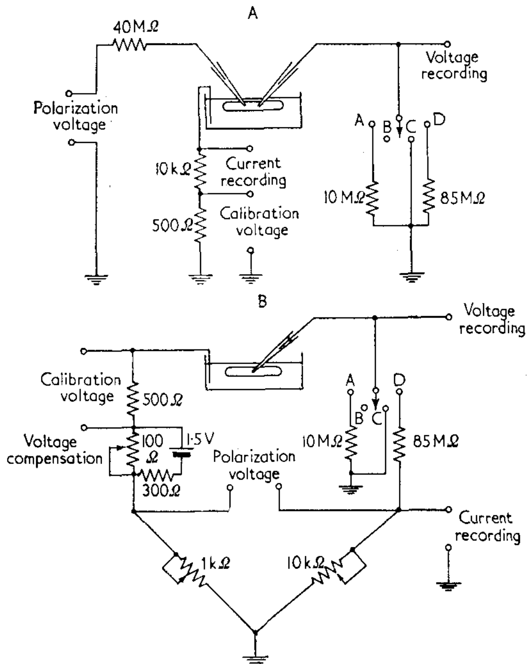

FIG. 2. Diagrams of polarization and recording with micro-electrodes. 2A, two micro-electrode method. Connexion A of the micro-switch, for measurement of electrode resistance; B, for membrane potential recording; C, for short-circuit; D, for adjustment of grid current. 2B, one micro-electrode method. Balancing the bridge is performed by adjusting resistances of 1 k Ω and 10 k Ω with the micro-switch of connexion D. A voltage is applied to compensate for small current passing through the membrane.

3. *Recording and stimulation*

When recording is made by means of micro-electrodes, care must be taken to improve high frequency response, because electrode resistance is very high. A fine Ag-AgCl wire dipped into the KCl solution of the micro-electrode makes an electrical connexion with the input of the pre-amplifier. A relatively large Ag-AgCl wire dipped into the bathing medium in the chamber serves as a ground lead. It is necessary to use a feed-back device in the pre-amplifier such as developed by Haapanen and

Ottoson (1954), MacNichol and Wagner (1954), and Solms *et al.* (1953), because the rate of rise of the action potential of insect axons is very high. The amplified resting and action potentials are displaced oscillographically.

In addition to external stimulation which produce propagated action potentials, stimulation by an impaling micro-electrode is very useful in examining various electrical properties. This is achieved either by passing current through a second micro-electrode inserted in the same axon less than 50 μ away from the first, or by passing current through the recording micro-electrode. In the latter case, however, a Wheatstone bridge circuit has to be used to prevent potential differences other than the membrane potential from being recorded. Figure 2 shows diagrams of stimulation and recording.

The physiological solution requires comment in view of the wide variation in ionic composition of hemolymph. This will be discussed in Section IV.

B. MEMBRANE RESTING AND ACTION POTENTIALS

1. *Measurements of resting and action potentials*

Measurements of the resting and action potentials have so far been made using cockroach giant axons (*Periplaneta americana*) exclusively. The first attempts by Boistel and Coraboeuf (1954) and Coraboeuf and Boistel (1955) gave an average resting potential of 78 mV and an average action potential of 85mV. Boistel (1960) discussed possible attentuation of the action potential caused by high electrode resistance, and supposed a value of 30–40 mV for the true overshoot and 70 mV for the resting potential. The author obtained an average resting potential of 64·5 mV and the average action potential of 92·2 mV in his first attempt to record them, the values for the action potential being compensated for possible distortion by calculation (Yamasaki and Narahashi, 1957c). In a later experiment, 70·3 mV and 94·5 mV were obtained for the average resting and action potentials respectively (Yamasaki and Narahashi, 1958b, 1959a). In this case, a feed-back amplifier was used to improve high frequency response, and the sodium concentration in Ringer's solution was raised from 159·6 mM, which was close to that used by Boistel (1960), to 214 mM. More recently, the values for the resting and action potentials were improved to 77 $\pm$ 0·7 mV (S.E. of mean, 24 measurements, range 70–83 mV) and 99 $\pm$ 1·4 mV (19 measurements, range 88–110 mV) respectively using the modified Ringer's solution (Narahashi and Yamasaki, 1960a). This seems to be the most reliable measurement available for the moment. An example of an action potential is illustrated in Fig. 3.

2. *Interpretation of resting and action potentials*

It has been well established in giant axons of the squid that the membrane potential can be described in terms of the membrane conductances for sodium and potassium and of the concentration gradients of these ions across the membrane (cf. Hodgkin, 1958). In the resting state the nerve membrane is more permeable to potassium than to sodium ions. Furthermore, the potassium concentration is much higher in the axoplasm than in the external medium or serum, while the situation is opposite in respect to

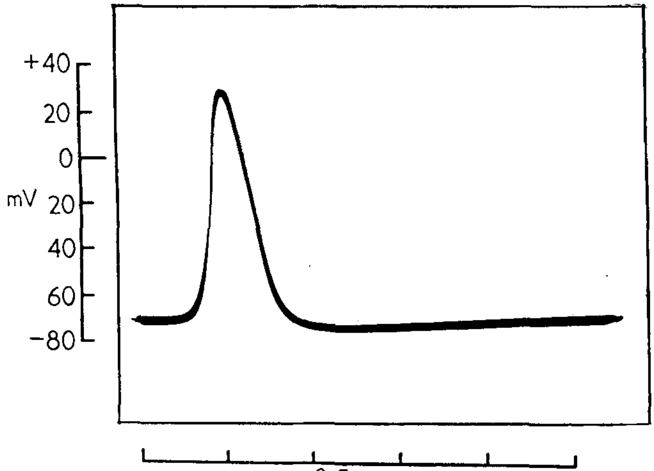

FIG. 3. Record of an action potential from the giant axon of the cockroach.

sodium. Thus, the membrane potential in the resting state approaches the equilibrium potential for potassium which is expressed as

$$E_K = \frac{RT}{F}\log_e\frac{[K]_o}{[K]_i} \tag{1}$$

where E_K is the potassium equilibrium potential, R is the gas constant, T is the absolute temperature, F is the Faraday, and $[K]_i$ and $[K]_o$ are potassium concentrations inside and outside the membrane. Upon excitation, however, the sodium conductance of the membrane is greatly and rapidly raised, so that the membrane potential now approaches the equilibrium potential for sodium which is expressed as

$$E_{Na} = \frac{RT}{F}\log_e\frac{[Na]_o}{[Na]_i} \tag{2}$$

where E_{Na} is the sodium equilibrium potential, and $[Na]_i$ and $[Na]_o$ are sodium concentrations inside and outside. Since the sodium equilibrium

potential has an opposite sign to the potassium equilibrium potential, the membrane potential is temporarily reversed in sign during excitation producing an overshoot or an active membrane potential.

However, the observed resting potential is usually smaller than the potassium equilibrium potential. This is because the permeabilities of the membrane to other kinds of ions are also involved. Thus, if we take into consideration the relative permeability to sodium, potassium and other ions, the resting potential can be expressed by the following equation which has been shown to fit the observation:

$$E_m = \frac{RT}{F}\log_e\frac{P_K[K]_o+P_{Na}[Na]_o+P_{Cl}[Cl]_i}{P_K[K]_i+P_{Na}[Na]_i+P_{Cl}[Cl]_o}. \tag{3}$$

E_m is the membrane potential, P_K, P_{Na} and P_{Cl} are relative permeabilities to potassium, sodium and chloride ions, and $[Cl]_i$ and $[Cl]_o$ are the chloride concentrations inside and outside. The ions which participate in producing the membrane potential other than sodium and potassium ions may be the one other than chloride.

The applicability of this theory to the membrane potential of the cockroach giant axons was examined. The potassium content of the sheathed nerve cord of the cockroach was estimated as 140 mM/l of tissue water (Tobias, 1948a). Using this figure, the potassium equilibrium potential is calculated as 97·7 mV from equation (1). However, since the potassium concentration of the hemolymph serum is estimated as 17·3 mM/l of tissue water (Tobias, 1948a), the actual concentration of potassium in the nerve fibers should be more than 140 mM/l of tissue water. Hence the true value for the potassium equilibrium potential is more than 97·7 mV. It follows that the observed resting potential is smaller than the potassium equilibrium potential by more than 20 mV. This low resting potential is undoubtedly responsible for the production of the positive phase following the spike potential, as will be discussed later (III, D, 2).

The concentration of sodium in the sheathed nerve cord of the cockroach is estimated as 83·9 mM/l of tissue water (Tobias, 1948a). The sodium equilibrium potential is then calculated as 24·0 mV from equation (2) in the modified Ringer's solution (214 mM Na^+). However, in this case, again, this value is slightly underestimated because the sodium concentration in the hemolymph serum is estimated as 107 mM (Tobias, 1948a) which is somewhat higher than that in the sheathed nerve cord. The observed active membrane potential or overshoot is 22 mV. It follows that the observed value is slightly smaller than the sodium equilibrium potential. This may be expected because at the peak of the action potential

the membrane conductances for ions other than Na are not zero (cf. equation (3)).

3. *Comparison with other nerves and muscles*

Examples of the magnitudes of the resting and action potentials are given in Table I. Although the values are somewhat different in each animal, the values from the cockroach giant axons fall in the range for other animals. It should be noted that in insects the resting potential is higher in nerve than in muscle, whereas the reverse relation is true for frogs.

TABLE I

Magnitudes of resting and action potentials from various excitable tissues

Animal	Tissue	Resting potential (mV)	Action potential (mV)	Reference
Loligo forbesi	Non-myelinated nerve	62	88	Hodgkin and Katz (1949a)
Loligo pealii	,,	61	104	Curtis and Cole (1942)
Sepia officinalis	,,	62	120	Weidmann (1951a)
Homarus vulgaris	,,	62	106	Hodgkin and Huxley (1945)
Homarus americanus	,,	73	101	Tobias and Bryant (1955)
Carcinus maenus	,,	71–94	116–153	Hodgkin and Huxley (1945)
Rana esculenta	Myelinated nerve	71	116	Huxley and Stämpfli (1951a)
Cat	Motoneurone	70	90–100	Brock, Coombs and Eccles (1952)
Toad	,,	50	70	Araki, Otani and Furukawa (1953)
Cat	Cortical neurone	87	80	Li (1955)
Rabbit	Superior cervical ganglion	65–80	70–96	Eccles (1955)
Rana temporaria	Striated muscle	88	119	Nastuk and Hodgkin (1950)
,,	,,	85	112	Nicholls (1956)
Frog and toad	,,	84	117	Furukawa (1953)

TABLE I—*cont.*

Animal	Tissue	Resting potential (mV)	Action potential (mV)	Reference
Oxya sp.	Wing muscle	50–70	*	Hagiwara (1953)
Gampsocleis burgeri	,,	60	†	Hagiwara and Watanabe (1954)
Locusta migratoria danica	,,	47	†	,,
Mecopoda Elongata, f. nipponensis	,,	41	†	,,
Platypleura kaempferi	Sound muscle	61	†	,,
Graptopsaltria nigrofuscata	,,	42	†	,,
Periplaneta americana	Leg muscle	*c.* 60	‡	Hoyle (1955)
Locusta migratoria migratorioides	,,	*c.* 60	‡	,,
,,	,,	60	§	del Castillo, Hoyle and Machne (1953)
Dog	Purkinje fiber	90	121	Draper and Weidmann (1951)
Kid	,,	94	135	,,
Calf and sheep	,,	98	132	Weidmann (1955b)

* Equal to or smaller than the resting potential.
† About 85% of the resting potential.
‡ No overshoot or overshoot by less than 15 mV.
§ Overshoot by less than 20 mV.

C. ELECTRICAL PROPERTIES

1. *Cable property of the axon*

The axoplasm, which contains various electrolytes and has a low electric resistance, is surrounded by a membrane of about 100 Å thick. The membrane shows a high electric resistance. Hence the axon resembles a cable as far as its passive electrical properties are concerned. Figure 4 shows a diagram of the electric component of a nerve fiber. The membrane has a capacitative component in parallel with a resistance and an e.m.f., whereas the axoplasm has only a resistance component.

When a pulse of constant current is passed through the membrane at any point of an axon which is bathed in a large volume of conductive

medium, the steady-state potential difference produced across the membrane is expressed by

$$\underset{t\to\infty}{V(\chi)} = \frac{I}{2}\sqrt{(\gamma_m \gamma_i)}\,e^{-(\chi/\lambda)}, \tag{4}$$

where V is the potential difference across the membrane, χ is the distance along the axon, t is time, I is the intensity of total current flowing through the membrane, γ_m is the resistance times unit length of the nerve membrane, γ_i is the resistance per unit length of the axoplasm and λ is the

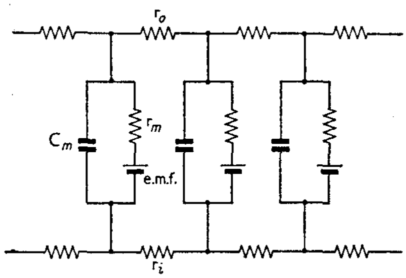

FIG. 4. Diagram of electric components of an axon. C_m, membrane capacity; e.m.f., electromotive force of the membrane; r_i, axoplasm resistance; r_m, membrane resistance; r_o, resistance of surrounding medium.

length constant. The length constant is related to the membrane resistance, the axoplasm resistance and the resistance of the external fluid by the following equation:

$$\lambda = \sqrt{\left(\frac{\gamma_m}{\gamma_i + \gamma_o}\right)} \tag{5}$$

where γ_o is the resistance per unit length of the external fluid. However, when the axon is bathed in a large volume of external fluid, as is the case when a micro-electrode is used, the resistance of the external fluid becomes negligible compared with the axoplasm resistance. Under such a condition

$$\lambda = \sqrt{\left(\frac{\gamma_m}{\gamma_i}\right)}. \tag{6}$$

When the membrane potential is recorded at the point where the current is passed through the membrane, equation (4) is simplified at the steady-state of the membrane potential. Thus

$$V = \frac{I}{2}\sqrt{(\gamma_m \gamma_i)}. \tag{7}$$

The term $\sqrt{(\gamma_m \gamma_i)}/2$ is called "effective membrane resistance". Equation (7) means that the recorded membrane potential is related to the membrane resistance and the axoplasm resistance if the current strength is kept constant. Further, if we assume that the axoplasm resistance undergoes no change, which is true under some conditions, the potential change is indicative of the membrane resistance. This condition can be established when a micro-electrode is inserted in an axon bathed in a large volume of conductive medium, and passing of current and recording of potential are made by the micro-electrode (Fig. 2B). Alternatively, we can insert two micro-electrodes in an axon, but the distance between them should be much shorter than the length constant of the axon (Fig. 2A).

The time course of potential change produced by passing a pulse of constant current through the membrane is expressed by

$$\underset{x=0}{V(t)} = \frac{I}{2}\sqrt{(\gamma_m \gamma_i)}\,\mathrm{erf}\sqrt{\left(\frac{t}{\tau_m}\right)}, \tag{8}$$

where τ_m is the time constant of the membrane.

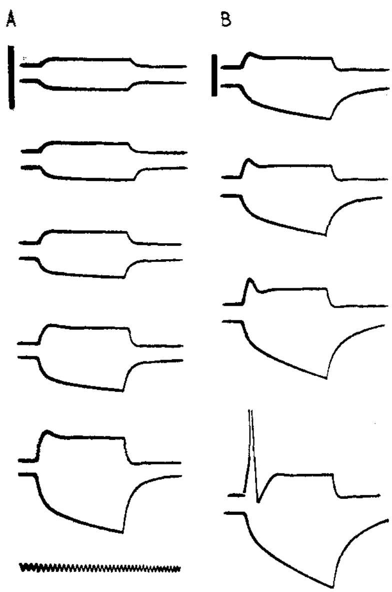

FIG. 5. Electrotonic potentials are produced by square pulses of polarizing current in the cockroach giant axon. Each pair of records shows a catelectrotonic (upward deflexion) and an anelectrotonic potential (downward deflexion) evoked by equal intensity of current. A, from top to bottom, current intensities are 3·52, 5·68, 7·49, 11·8 and 20·2 × 10^{-9} A. Voltage calibration, 10 mV. Time calibration of 1000 c/s applies to both A and B. B, current intensities, 27·5, 35·2, 37·8 and 45·3 × 10^{-9} A. Voltage, 20 mV. The action potential in the bottom pair is truncated (from Yamasaki and Narahashi, 1959b).

2. *Voltage-current relation*

Two micro-electrodes were inserted in an axon less than 50 μ apart, the distance being much shorter than the length constant. Pulses of current were passed through one electrode, while the membrane potential was recorded by the other (Narahashi, 1961a). Sometimes one micro-electrode was used both for polarization and for recording. In the latter case, the Wheatstone bridge circuit of Araki and Otani (1955) and Frank and

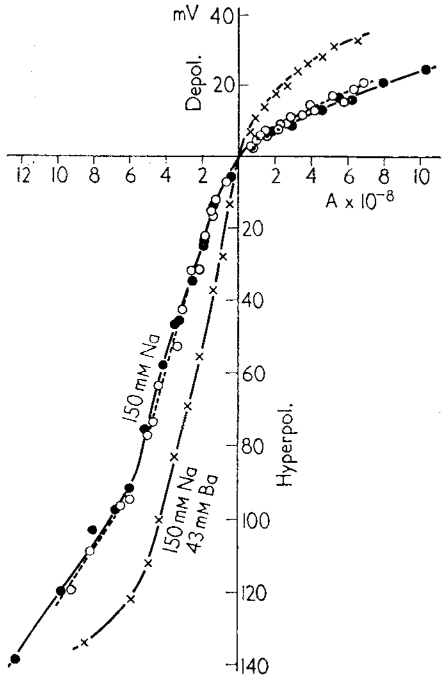

FIG. 6. Voltage-current relations in the cockroach giant axon. Measurements were made at first in 150 mM Na (●), next in 150 mM Na + 43 mM Ba (×), and then again in 150 mM Na (○). The abscissa represents the current strength and the ordinate the steady-state potential (from Narahashi, 1961a).

Fuortes (1956) was employed in order to eliminate the potential drop across resistances other than the membrane (Yamasaki and Narahashi, 1959b). A series of records of the electrotonic potential is illustrated in Fig. 5. When the intensity of current is weak, the catelectrotonic and the anelectrotonic potentials are symmetrical. With stronger current, however, the steady-state magnitude of the catelectrotonic potential becomes smaller than the anelectrotonic potential, and there appears a hump at the initial phase of the catelectrotonic potential. The hump grows with

increasing current intensity, and an action potential is finally produced from the summit of the hump. On the other hand, no action potential is elicited during or on stopping an anodal current. The hump is produced partly by rectification and partly by the local response. The relationship between the current intensity and the steady-state magnitude of the electrotonic potential is shown in Fig. 6, the data for which are taken from an experiment other than that of Fig. 5. It is clearly seen that membrane resistance becomes smaller when the membrane is depolarized. It should be noted that though the membrane resistance increases upon moderate hyperpolarization, it decreases when the hyperpolarization exceeds a certain level.

The voltage–current relation shown in Fig. 6 is almost the same as that obtained from other kinds of nerve. The rectification by cathodal current has been shown in many kinds of nerves, e.g. in squid giant axons (Cole and Curtis, 1941), lobster giant axons (Narahashi, unpublished) and in myelinated nerve fibers of the frog (Stämpfli, 1959). However, rectification by strong anodal current has so far received less attention (spinal ganglion cells, Ito, 1957).

3. *Critical depolarization*

When depolarization by cathodal current attains a certain critical level, an action potential appears. The critical depolarization ranges from 15 mV to 29 mV, having a mean value of 22 mV at 16–23·5° C (mean 20°C) (Yamasaki and Narahashi, 1959b). At 28–32·5°C (mean 30·2°C), a mean value of 25 mV has been obtained (Narahashi, 1961a). The threshold current passing through the impaling micro-electrode was estimated as $4{\cdot}42 \times 10^{-8}$ A in the former study, and $3{\cdot}4 \times 10^{-8}$ A in the latter.

4. *Membrane electrical constants*

It is possible to estimate the effective membrane resistance by measuring current intensity and the steady-state electrotonic potential (equation 7). However, it is necessary to make some assumptions with regard to the axoplasm resistance and the diameter of the axon in order to calculate the specific membrane resistance unless the electrotonic potential is recorded at several points along the axon. With the value of the specific membrane resistance, the specific membrane capacity can be estimated from the time constant of the membrane by

$$\tau_m = R_m C_m, \tag{9}$$

where τ_m is the membrane time constant, R_m is the specific membrane resistance, and C_m is the specific membrane capacity. Thus the mean

values for the membrane constants are estimated as follows at 20°C (Yamasaki and Narahashi, 1959b).

Effective membrane resistance	283 KΩ
Specific membrane resistance	800 Ω cm^2
Specific membrane capacity	6·3 μF cm^{-2}
Membrane time constant	4·2 msec
Membrane length constant	0·86 mm

The electrical constants of other kinds of nerve and muscle membranes are given in Table II. It will be seen that the membrane capacity of the cockroach giant axon is larger than that of any other.

TABLE II

Membrane electrical constants of nerves and muscles

Tissue	Fiber diameter (μ)	R_m (Ωcm^2)	R_i (Ωcm)	C_m (μF cm^{-2})	τ_m (msec)	λ (mm)	Reference
Squid axon (*Loligo*)	500	700	30	1·0	0·7	6·0	Cole and Hodgkin (1939) Hodgkin, Huxley and Katz (1949)
Squid axon (*Sepia*)	200	9200	60	1·2	14	5·7	Weidmann (1951a)
Crab axon (*Carcinus*)	30	7700	90	1·1	6.8	2·0	Hodgkin (1947a)
Lobster axon (*Homarus*)	75	2300	60	1·3	2·3	1·6	Hodgkin and Rushton (1946)
		2400	95	0·8		1·7	Tobias (1960)
Frog muscle (*Rana*)	45	4300	230	5	19	1·1	Katz (1948)
Crab muscle (*Portunus* and *Carcinus*)	180	120	70	42	4·6	0·9	Fatt and Katz (1953)
Kid Purkinje fiber	75	1900	110	12	20	1·9	Weidmann (1952)

5. *Velocity of conduction*

Conduction velocity has been measured in several insects. Table III summarizes the results.

TABLE III

Velocity of conduction

Insect	Nerve	Diameter (μ)	Conduction Velocity (m/sec)	Reference
Periplaneta americana	Giant axon	45	6	Roeder (1948a)
,,	,,	45	9–12	Roeder, Kennedy and Samson (1947)
,,	,,		6·6–7·2	Boistel and Coraboeuf (1954)
,,	,,	50	7	Boistel (1960)
,,	,,		5–6	Pumphrey and Rawdon-Smith (1937)
,,	Cercal nerve		1·5–2·0	Roeder (1948a)
,,	,,		,,	Roeder, Kennedy and Samson (1947)
,,	,,		1·5	Pumphrey and Rawdon-Smith (1937)
Anax imperator (nymph)	Giant axon	12–16	3·5–4·5	Fielden (1960)
,,	Lateral nerve (N5)		1·5–3·0	,,
Locusta migratoria	Giant axon	8–16		Cook (1951)
,,	,,		3–4	Fielden (1960)
Locusta migratoria migratorioides	Crural nerve		2·2—2·7	Hoyle (1953)
,,	Crural nerve (axon F)	7–13	2·2	Hoyle (1954b)
,,	Crural nerve (axon S_1)	7–12	2·3	,,
,,	Crural nerve (axon S_2)	5–6	1·6	,,
Allomyrina dichotomus	peripheral nerve		0·85–1·07	Yamasaki and Ishii (1952a)

6. *Refractory period*

The relative and absolute refractory periods are summarized in Table IV.

7. *Effects of sodium and potassium on membrane potential*

In the foregoing section (III, B, 2), the possibility of describing the resting and action potentials in terms of potassium and sodium conductances and concentration gradients across the membrane is discussed. In

TABLE IV

Refractory period

Insect	Nerve	Relative refractory period (msec)	Absolute refractory period (msec)	Reference
Locusta migratoria migratorioides	Crural nerve (axon F)	15	2·6	Hoyle (1954b)
,,	Crural nerve (axon S_1)	15	2·6	,,
,,	Crural nerve (axon S_2)	15	3·0	,,
Locusta migratoria	Nerve cord	8	3·2	Boistel (1960)
Periplaneta americana	Nerve cord	13	2	,,

order to evaluate this view, the effect of changing the external concentrations of potassium and sodium on the membrane potential was examined (Yamasaki and Narahashi, 1958b, 1959a).

Increasing external potassium causes depolarization, whereas decreasing it has little, if any, effect on the resting potential. When the resting potential is plotted against the logarithm of the external potassium concentration, the measurements at potassium concentrations higher than about 20 mM fall on a straight line (Fig. 7). If the resting potential were

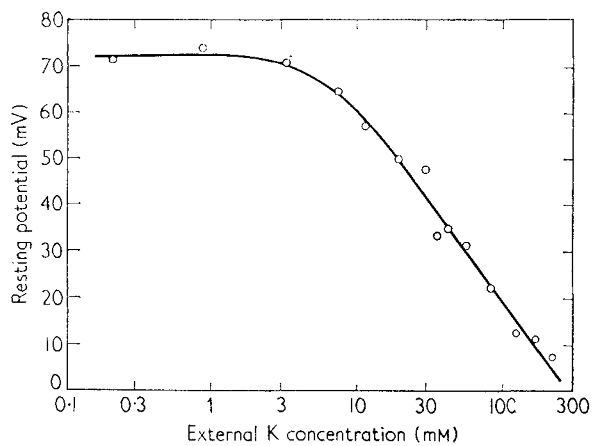

FIG. 7. Effect of varying the external concentration of potassium (abscissa) on resting potential (ordinate) in the cockroach giant axon (from Yamasaki and Narahashi, 1959a).

solely determined by the potassium equilibrium potential, it would change by 59 mV for a tenfold change in potassium concentration (equation 1). However, the slope in Fig. 7 shows a 42 mV change in resting potential instead of a 59 mV change. This implies that the conductances to ions other than potassium participate to some extent in generating the potential, and is in keeping with the fact that the observed resting potential is smaller than the potassium equilibrium potential. The

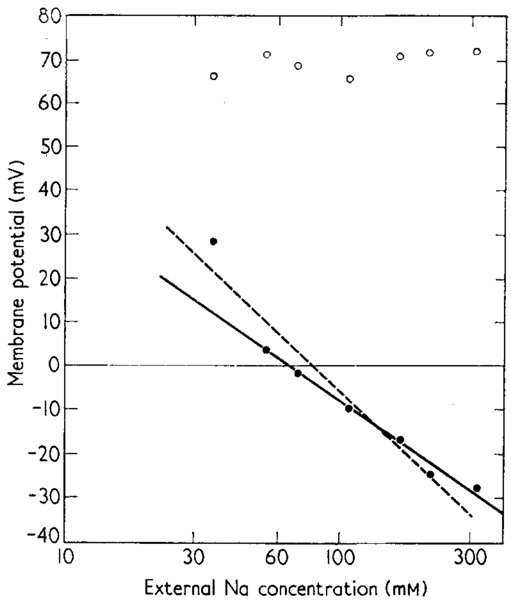

FIG. 8. Effect of varying the external concentration of sodium (abscissa) on resting potential (open circles) and active membrane potential (filled circles) (ordinate) in the cockroach giant axon. The broken line is drawn according to equation (2), which has a slope of 59 mV for a tenfold change in sodium concentration. The solid line is drawn through the observed values of the active membrane potential excluding the lowest value (from Yamasaki and Narahashi, 1959a).

same holds true for the squid giant axon (Curtis and Cole, 1942), the frog sartorius muscle (Ling and Gerard, 1950), the frog myelinated nerve (Huxley and Stämpfli, 1951b) and for the lobster and crayfish giant axons (Dalton, 1958, 1959; Narahashi, unpublished). Excitability is lost when the potassium concentration is raised beyond a certain level.

Change in external sodium concentration has a quite different effect on the membrane potential. When its concentration is lowered by substituting choline for it, the magnitude of the action potential is effectively reduced, while the resting potential undergoes little change (see also

Boistel, 1960). Impulse conduction is impaired when the sodium concentration is lowered beyond a certain critical point. These effects are reversible on washing with normal Ringer's solution. In Fig. 8 the resting and action potentials are plotted against the logarithm of the external sodium concentration. The measurements of the action potential fall on a straight line. If the membrane behaved as a perfect sodium electrode during the action potential, it would change by 59 mV for a tenfold change in external sodium (equation 2). However, the slope of the straight line observed is less than that. Therefore conductances of ions other than sodium participate in determining the peak value for the action potential, and this is in keeping with the fact that the observed action potential is smaller than the sodium equilibrium potential.

A similar effect of Na-deficient media on the action potential has been observed in many other nerves and muscles (e.g. frog myelinated nerve fibers, Huxley and Stämpfli, 1951b; frog muscle fibers, Nastuk and Hodgkin, 1950; mammalian Purkinje fibers, Weidmann, 1955a; squid giant axons, Hodgkin and Katz, 1949a; crayfish giant axons, Dalton, 1959; lobster giant axons, Dalton, 1958; Narahashi, unpublished).

8. *Mechanism of action potential production*

The behavior of the action potential in Na-deficient media strongly supports the view that the rising phase of the action potential is produced by a regenerative rise in sodium conductance. It was also found that during the course of blockade by perfusion with Na-deficient media the rising phase of the action potential is reduced much faster than the falling phase (Yamasaki and Narahashi, 1959a). This finding also suggests the importance of sodium ions for the rising phase of the action potential.

It is reasonable to assume that the "ionic theory" is applicable to the cockroach giant axon. The ionic theory is summarized as follows (Hodgkin, 1951, 1958; Hodgkin and Huxley, 1952a–d; Hodgkin *et al.*, 1952): The membrane potential in the resting state is mostly determined by the potassium conductance and the potassium concentration gradient, approaching the potassium equilibrium potential. When the membrane is depolarized by a cathodal current, the sodium conductance is greatly raised. If the intensity of the cathodal current is sufficiently large, the sodium conductance rise progresses regeneratively, so that the membrane is depolarized and approaches the sodium equilibrium potential. This forms the rising phase of the action potential. Following this, however, the potassium conductance also rises with a certain delay, while the sodium conductance that has once been raised is now lowered to the resting level. These two bring the membrane potential back toward the

resting level, forming the falling phase of the action potential. Therefore, during an action potential sodium ions flow into the axoplasm while potassium ions flow out. The changes in ionic concentrations in the axoplasm are restored metabolically after activity.

9. *Ionic current associated with an action potential*

It is possible to calculate the ionic current during an action potential from the values of the membrane electrical constants. The total quantity of sodium entering the axon during excitation is given by $C_m V/F$ (Hodgkin and Katz, 1949a). With a C_m value of 6·3 μF cm^{-2} (Yamasaki and Narahashi, 1959b) and a spike height of 99 mV (Narahashi and Yamasaki, 1960a), the total quantity of sodium entering the axon is calculated as $6{\cdot}3 \times 10^{-12}$ M cm^{-2} impulse^{-1} for the cockroach axon. However, the true value may be larger than this because (1) sodium entry during the rising phase may be partly masked by potassium egress, and (2) sodium entry may continue during the falling phase. This is actually true in other nerves, differing by as much as 100%. For example, the observed values are $3{\cdot}5 \times 10^{-12}$ M cm^{-2} impulse^{-1} for *Loligo* axons and $3{\cdot}7$–$3{\cdot}8 \times 10^{-12}$ M cm^{-2} impulse^{-1} for *Sepia* axons (Keynes, 1951; Keynes and Lewis, 1951) as against the calculated values of $1{\cdot}4$–$1{\cdot}6 \times 10^{-12}$ M cm^{-2} impulse^{-1} for *Loligo* axons and $1{\cdot}55 \times 10^{-12}$ M cm^{-2} impulse^{-1} for *Sepia* axons (Hodgkin, 1951; Hodgkin and Katz, 1949a; Keynes and Lewis, 1951). It follows that the sodium entry in the cockroach giant axons may be as large as $1{\cdot}5 \times 10^{-11}$ M cm^{-2} impulse^{-1}.

Under conditions which allow the action potential to be propagated along the axon bathed in a large volume of a conducting medium, the ionic current at the moment when the rate of rise of the action potential is at a maximum can be calculated from the membrane capacity and the maximum rate of rise of the action potential (Hodgkin and Katz, 1949a; Narahashi, 1961a; Yamasaki and Narahashi, 1959b). The total current passing through the membrane is composed of a capacity current and an ionic current, I_i. Thus we have

$$I = C_m \frac{dV}{dt} + I_i. \tag{10}$$

On the other hand, the total membrane current is also expressed as

$$I = \frac{a}{2R_i\theta^2} \frac{d^2 V}{dt^2} \tag{11}$$

where a is the radius of the axon, and θ is the velocity of conduction. Hence

$$I_i = \frac{a}{2R_i\theta^2} \frac{d^2 V}{dt^2} - C_m \frac{dV}{dt}. \tag{12}$$

It follows that at the moment when the rate of rise of the action potential is at a maximum, equation (12) is simplified as follows:

$$I_i = -C_m \frac{dV}{dt}. \tag{13}$$

When a membrane capacity of 6·3 μF cm^{-2} (Yamasaki and Narahashi, 1959b) and a maximum rate of rise of 1100 V/sec (Yamasaki and Narahashi, 1959a), the inward ionic current at the moment when the rate of rise is at a maximum is calculated as 6·9 mA cm^{-2}. This value is greater than that of other nerves and muscles, i.e. 0·6–1·0 mA cm^{-2} for squid axons (Hodgkin *et al.*, 1949, 1952; Hodgkin and Katz, 1949a), 1·29 mA cm^{-2} for crab muscle (Fatt and Katz, 1953), and 2 mA cm^{-2} for frog muscle (Nastuk and Hodgkin, 1950).

10. *Effect of displacement of membrane potential on action potential*

It has been established for the squid giant axon that the ability of the membrane to undergo sodium conductance rise is decreased by lowering the membrane potential and is increased by raising it (Hodgkin and Huxley, 1952c). This has also been shown to be true for Purkinje fibers (Weidmann, 1955a, b).

In cockroach giant axons, lowering the membrane potential by cathodal current depresses the rise of sodium conductance, whereas raising the membrane potential has little effect on it (Narahashi, 1961a; Yamasaki & Narahashi, 1959b). In these studies, the propagated action potential was observed under polarization, so that the maximum rate of rise was taken as a measure of the inward sodium current. The active membrane potential attains the normal level when the membrane is hyperpolarized within limits, whereas it becomes smaller when the membrane is depolarized. It is therefore concluded that the ability of the membrane to undergo sodium conductance rise is maximum at the normal resting potential. This situation is different from that of squid axons, as noted earlier, in which the ability is not maximal at the resting potential.

11. *Effect of calcium on resting and action potentials*

There is general agreement in many kinds of excitable tissues that the presence of calcium ions in the external medium is necessary for normal maintenance of excitability (cf. Brink, 1954; Shanes, 1958a, b).

When the external calcium concentration is raised to 18mM from the normal value of 1·8 mM, the resting potential is increased by about 3mV. The active membrane potential is also increased slightly, while the maximum rate of rise of the action potential is decreased by about 20–30%.

The maximum rate of fall undergoes little change. The configuration of the action potential is changed, the onset of the falling phase being somewhat delayed. A decrease in calcium concentration has an opposite effect on the resting potential and the active membrane potential, decreasing both. However, the maximum rate of rise of the action potential changes in the same direction, as in the case of high-Ca media, being slowed down. The maximum rate of fall is also decreased. When the external calcium is completely removed, the membrane is depolarized and conduction is finally blocked. All these effects including those of high-Ca and low-Ca media are reversible on washing with normal Ringer's solution, unless the axon is left in the test medium for a long time.

It is of interest to compare these responses of the cockroach giant axons to altered calcium concentrations with those of other nerves and muscles in view of the importance of calcium in excitation. This is especially important from the point of view of insect toxicology, because most insecticides are neurotoxins, and because there is evidence suggesting an intimate relation of calcium to the action of some insecticides on nerve (Gordon and Welsh, 1948; Welsh and Gordon, 1947). First of all, the membrane potential of the cockroach giant axons behaves in altered calcium concentrations much as in other nerves, i.e. the resting and action potentials become smaller in low calcium concentration. Secondly, the increase in the maximum rate of rise of the action potential is also observed in the squid giant axon (Shanes *et al.*, 1959). This is to be expected from the voltage-clamp data showing the increase in the maximum sodium conductance by a rise in calcium concentration (Shanes *et al.*, 1959); however, another experiment shows a lack of influence of external calcium on maximum sodium conductance (Frankenhaeuser and Hodgkin, 1957). The maximum rate of fall of the action potential is accelerated more than the rate of rise by elevating calcium concentration in the squid giant axons (Shanes *et al.*, 1959), this result being different from that with cockroach giant axons.

Recent experiments on lobster giant axons have demonstrated that although the maximum rate of rise of the action potential is reduced by lowering calcium concentration, it is raised beyond the normal level upon hyperpolarization (Narahashi, unpublished). Furthermore, excitability of the axon poisoned by tetrodotoxin is partly restored by anodal polarization when the external calcium concentration is reduced. Tetrodotoxin is thought to suppress the sodium-carrying system (Narahashi *et al.*, 1960) and the excitability of the tetrodotoxin-poisoned axon cannot be restored by anodal polarization if bathed in normal calcium concentration. These results together with other available evidence lead us to

the hypothesis that calcium ions play a role in integrating the membrane molecular structure and are temporarily displaced from their original site upon stimulation, thereby causing a rise in membrane conductance (Tobias *et al.*, 1962). It is desirable to extend this sort of study to insect axons in view of the possible interaction of calcium with the action of insecticides.

It is noteworthy that the cockroach giant axon does not produce repetitive responses even in low-Ca media (Narahashi and Yamasaki, 1960a); nor does prolonged cathodal depolarization of 2–3 times threshold produce repetitive discharge (Yamasaki and Narahashi, 1959b), either in normal Ringer solution or in a low-Ca medium. In certain other types of nerve, lowering calcium concentration is known to elicit repetitive discharge in response to a single shock (cf. Brink, 1954; Shanes, 1958b). However, when the cockroach axons are treated with DDT, repetitive discharge is elicited by a single shock and lowering calcium concentration accelerates it (Gordon and Welsh, 1948; Welsh and Gordon, 1947). Furthermore, smaller axons in the cockroach abdominal nerve cord respond repetitively to a strong cathodal polarization, and the repetitive response is augmented by treatment with DDT (Yamasaki and Ishii, 1954c, 1957d). In view of the fact that many insecticides produce repetitive response in insect nerves, repetitive excitability will be discussed in more detail in a later section in connexion with the action of insecticides (VI).

12. *Effect of barium on resting and action potentials*

Barium ions can substitute for sodium ions to produce action potentials in crustacean muscle fibers (Fatt and Ginsborg, 1958) and in mammalian B and C, but not in A, fibers (Greengard and Straub, 1959). Barium ions also convert graded to all-or-none responses when applied to muscle fibers of the grasshopper, *Romalea microptera* (Werman *et al.*, 1961), or of the lobster, *Homarus americanus* (Werman and Grundfest, 1961). In the light of these observations, it is interesting to see the effect of barium ions on the cockroach giant axons (Narahashi, 1961a).

When bathed in isotonic barium solution in which sodium is replaced by barium keeping the other ions unchanged, cockroach giant axons become inexcitable immediately, but the effect is reversible on washing with normal Ringer's solution. However, when sodium is present at appropriate concentration, e.g. 100 mM or 150 mM, barium produces a marked change in configuration of the action potential. An example of a series of records of the action potential under the influence of barium is shown in Fig. 9. Column A shows the spike phase of the action potential, while column B is recorded with a slower sweep in order to show the slow

phase as well. The rising and falling phases of the action potential are slowed down by barium, the change becoming more conspicuous with increasing barium concentration. Parallel with this, the positive phase following the spike disappears and the negative after-potential is markedly augmented and prolonged. The latter change will be discussed in the section on after potentials (III, D, 3). The resting potential falls to

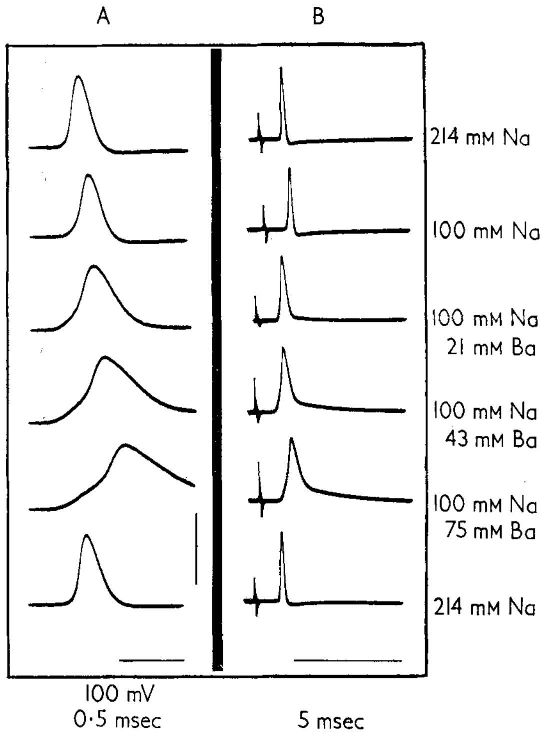

FIG. 9. Records of the action potential from the cockroach giant axon in various concentrations of barium. Both fast (left-hand column) and slow (right-hand column) sweep records are shown in each solution (from Narahashi, 1961a).

a small extent. These effects of barium are completely reversible on washing with normal Ringer's solution.

One of the causes of slowing the rate of rise of the action potential is probably the slight depolarization observed, since in normal Ringer's solution depolarization slows down the rising phase. Figure 10 shows the effect of displacement of the membrane potential by applied current on the action potential before and after treatment with barium. In normal

Ringer's solution, the total spike height increases linearly with hyperpolarization indicating a constant level of the active membrane potential, whereas it decreases with the depolarization more than the amount of depolarization. After treatment with barium, however, a linear relation between the displacement of the membrane potential and the total spike height obtains for the depolarized membrane. More marked effect is seen in the maximum rate of rise. In normal Ringer's solution, the rate of rise undergoes little change or is decreased upon hyperpolarization, whereas it is effectively decreased upon depolarization. After treatment with

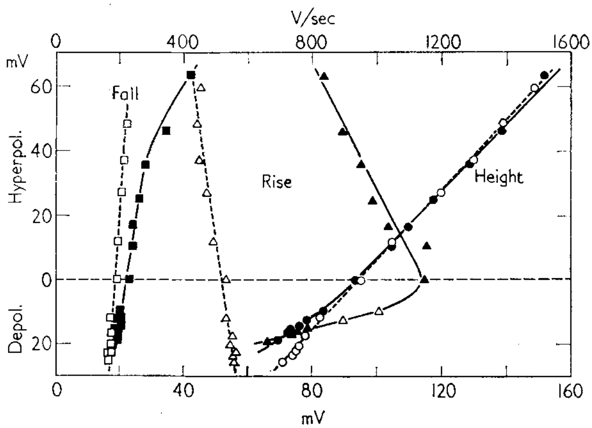

Fig. 10. Changes in the height (○ ●), and in the maximum rates of rise (△ ▲) and fall (□ ■) of the action potential produced by the displacement of the membrane potential from the resting level in the cockroach giant axon. The filled symbols represent the measurements in normal Ringer's solution, and the open ones in 150 mM Na + 43 mM Ba (from Narahashi, 1961a).

barium, it is almost stabilized at a low value, being unaffected by displacement of the membrane potential, as shown in Fig. 10. Since the maximum rate of rise of the action potential is indicative of the inward sodium current at the moment when the rate of rise is maximum (equation 13), the stabilization of the rate of rise at a low value indicates that the ability of the membrane to increase its sodium conductance is depressed and stabilized. Inactivation is no longer produced by depolarization, and no reactivation occurs on hyperpolarization. The experiment shown in Fig. 10 excludes the possibility that the slowing of the rate of rise of the action potential by barium is due to the slight depolarization observed.

Depression of the sodium-carrying system by barium would naturally lead to a rise in threshold and critical depolarization. This has been shown. In a Ringer's solution whose sodium concentration is lowered to 150 mM,

the average critical depolarization needed for firing is estimated to be 31 mV, while in a Ringer's solution containing 150 mM Na and 43 mM Ba it is raised to an average of 40 mV. However, the threshold current undergoes little change; it is estimated as $6{\cdot}3 \times 10^{-8}$ A in 150 mM Na, and $5{\cdot}9 \times 10^{-8}$ A in 150 mM Na plus 43 mM Ba. This means that barium increases membrane resistance. Analysis of the voltage–current relation (Fig. 6) reveals that the effective membrane resistance is raised by barium from the normal value of 930 kΩ to 1990 kΩ on the average.

13. *Effect of lithium on resting and action potentials*

Since an early study by Overton (1902), it has been known that lithium can be substituted for sodium in producing action potentials (e.g. Hodgkin, 1951; Keynes and Swan, 1959). This was demonstrated to be true for cockroach giant axons; no apparent change was observed in resting and action potentials when sodium was completely replaced by lithium (Narahashi, unpublished).

D. AFTER-POTENTIALS

In many kinds of nerves and muscles, an action potential terminates in a slow phase which is called "after-potential". In some cases two or three kinds of after-potential follow the action potential. Sometimes, a term "spike" or "spike potential" is used to distinguish the quick phase from the slow phase. The after-potentials, as well as the spike potential, have long received considerable attention, but it was not until the intracellular recording technique became available that the after-potentials could be interpreted in terms of changes in membrane conductances and ionic concentrations.

In the giant axons of the squid, the spike potential is followed by a positive phase or an undershoot which in turn is followed by a negative after-potential (Frankenhaeuser and Hodgkin, 1956). During the falling phase of the action potential potassium conductance increases, so that the membrane potential is temporarily increased beyond the normal resting level, forming a positive phase. The membrane potential returns to the resting level as the potassium conductance returns to normal. The rise in potassium conductance during the falling phase of the spike causes an outflow of potassium. The extruded potassium is retained near the axon membrane for some time, producing a small depolarization; this results in the negative after-potential. The same explanation is shown to be applicable to non-myelinated nerve fibers of the rabbit (Greengard and Straub, 1958). On the other hand, the negative after-potential of frog muscle

fibers directly follows the spike phase, and may be accounted for by a passive repolarization of the membrane (cf. Frank, 1957; Narahashi, 1960). In the crayfish and lobster giant axons, the spike potential is directly terminated in a negative after-potential, without showing a positive phase (Narahashi, unpublished; Watanabe and Grundfest, 1961), but no attempt has been made so far to explain the negative after-potential.

The after-potentials have received attention from the pharmacological point of view, because they are in many cases more sensitive to ions and drugs than the spike potential (cf. Shanes, 1958b). Changes in after-potentials by drugs are especially important in the case of insect nerve, because certain insecticides have been shown to affect the after-potentials seriously (Narahashi, 1960; 1962a, b; Narahashi and Yamasaki, 1960b, c). The insect toxicological aspect of after-potentials will be discussed in Section VI. The mechanism of production of the normal after-potentials has been explored in the cockroach giant axons, and will be described below (Narahashi and Yamasaki, 1960a).

1. *Mechanism of after-potential production*

a. Normal configuration of after-potentials. In normal Ringer's solution, the spike potential from the cockroach giant axon is followed by a positive phase of a few millivolts magnitude which is in turn followed by a negative after-potential (Fig. 11).

b. Effect of temperature on after-potentials. Raising the temperature causes an increase in the magnitude of the negative after-potential. When

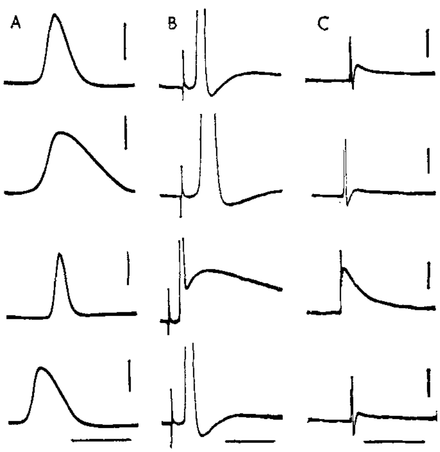

FIG. 11. Effect of changing the temperature on the action potential of the cockroach giant axon. Three records were taken at each temperature with different gain and sweep speed. Calibration: A, 50 mV, 1 msec; B, 5 msec; C, 5 mV (applies to B also), 50 msec. Temperature: from top to bottom, 20°C, 11°C, 32°C, 20°C.

the temperature is raised beyond a certain critical point, the whole slow phase is elevated so that the positive phase is seen only as a dip between the spike potential and the negative after-potential. A series of records is illustrated in Fig. 11, and the measurements are graphically shown in Fig. 12. The resting potential is increased by a rise in temperature; this is to be expected from equations (1) and (3), and is in accordance with the observation by Boistel (1960). A similar change was observed with lobster giant axons (Narahashi, unpublished), frog muscle fibers (Ling

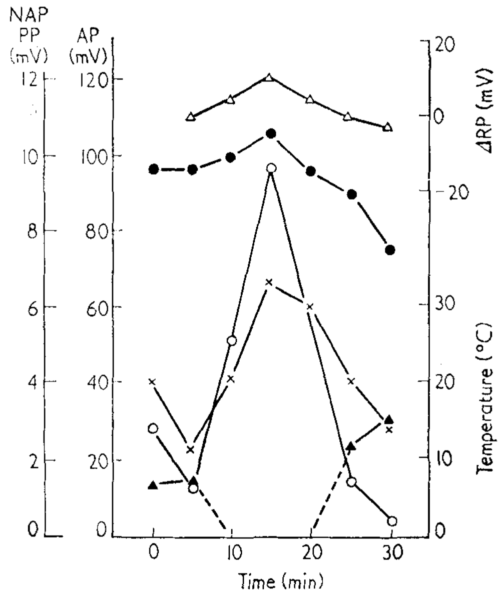

FIG. 12. Effects of temperature change (×) on the magnitude of the action potential (AP, ●), the initial height of the negative after-potential (NAP, ○), the magnitude of the positive phase (PP, ▲), and the resting potential (RP, △), in the cockroach giant axon.

and Woodbury, 1949) and cardiac muscle fibers (Coraboeuf and Weidmann, 1954; Trautwein *et al.*, 1953). The change is, however, very small in the squid giant axons (Hodgkin and Katz, 1949b). The total spike height is also increased, but this is almost entirely attributed to the rise in resting potential. The amplitude of the negative after-potential undergoes a larger percentage change upon altering temperature (see also Boistel, 1960). However, it should be noted that the critical temperature at which the positive phase converts its sign is greatly different from axon to axon. For example, in Fig. 12, the critical temperature is somewhere between 20°C and 30°C, while in the axons in Figs. 1 and 3 (Fig. 9 of the

present article) (Narahashi, 1961a) the critical temperature is more than 30°C. Likewise, the critical temperature is between 20°C and 30°C in Fig. 36 in Boistel (1960), while it is about 15°C in Fig. 37 in the same paper.

c. Effect of displacement of membrane potential on after-potential. The after-potentials undergo a marked change upon displacement of the membrane potential (Yamasaki and Narahashi, 1959b). Upon polarizing the membrane by applied current the amplitude of the positive phase increases by the same amount as the depolarization, and decreases by the same amount as the hyperpolarization (Fig. 13). Therefore, when the membrane is hyperpolarized by the same amount as the positive phase,

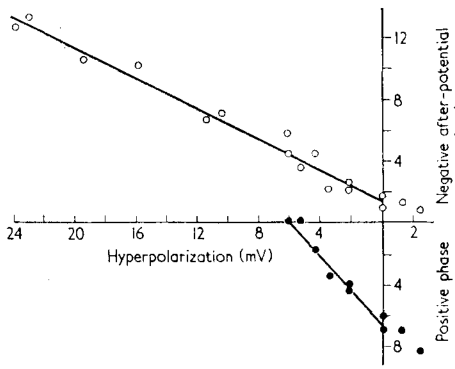

FIG. 13. The relations between the displacement of the membrane potential (abscissa) and the magnitude of the negative after-potential (○) and the positive phase (●) in the cockroach giant axon (from Yamasaki and Narahashi, 1959b).

the latter disappears and converts its sign on further hyperpolarization. On the contrary, the negative after-potential decreases upon depolarization and increases upon hyperpolarization (Fig. 13).

The high sensitivity of the after-potentials to displacement of the membrane potential should be taken into consideration whenever the former undergoes changes. Thus the effect of temperature on the after-potentials described before (III, D, 1b) is at least in part due to the change in resting potential with change in temperature.

d. Interpretation of positive phase. The positive phase is explained by a temporary rise in potassium conductance during the falling phase of the action potential. Since the resting potential is somewhat lower than the equilibrium potential for potassium (equation 1), a rise in potassium conductance during the falling phase of the action potential brings the membrane potential to a high value, thereby producing a positive phase. This explanation is in keeping with the observation on the relation between

the positive phase and the membrane potential described in the preceding section (III, D, lc).

e. Time course of negative after-potential. The length constant of the cockroach giant axon is estimated as 0·86 mm (Yamasaki and Narahashi, 1959b) and the conduction velocity is 6–7·2 msec (Boistel and Coraboeuf, 1954; Roeder, 1948a). Hence it takes only about 0·1msec for an impulse to travel a distance of one length constant. It follows that the conditions are close to those obtained with uniform polarization in so far as the slow

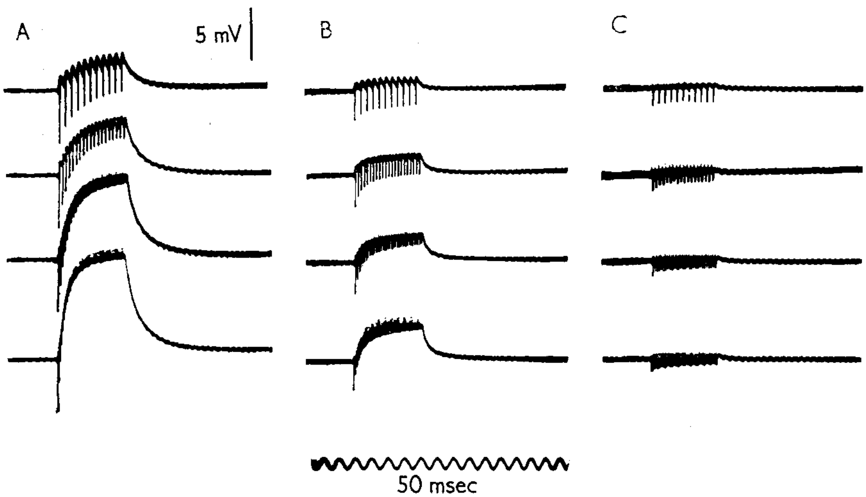

FIG. 14. Trains of impulses at varying frequencies recorded in different concentrations of potassium. Cockroach giant axon. Only the positive phase and the negative after-potential are seen; the spike potential is too large to be recorded. A, in 0 mM K; B, in 3·1 mM K; C, in 10·0 mM K. Frequencies of impulses are, from top to bottom in each column, 50, 100, 150 and 200 c/s (from Narahashi and Yamasaki, 1960a).

phase of the action potential is concerned. Therefore, any declining phase of the potential would decay exponentially if that phase were passive in nature (cf. Hodgkin and Rushton, 1946). It was found that the falling phase of the negative after-potential of the cockroach giant axon can be expressed as a single exponential term with a mean time constant of 9·2 msec (Narahashi and Yamasaki, 1960a). Therefore, the falling phase is thought to be passive in nature.

f. Effect of repetitive stimulation and potassium concentration on after-potentials. Figure 14 shows a build-up of the negative after-potential by repetitive stimuli in various concentrations of potassium. Lowering the potassium concentration causes an increase in the positive phase and the negative after-potential. The build-up of the negative after-potential is

more conspicuous in lower potassium concentration than in higher ones. It is also known that the membrane is depolarized by raising potassium concentration (Yamasaki and Narahashi, 1959a). In view of these observations, together with the passive decay of the negative after-potential (III, D, le), the negative after-potential can be explained by an accumulation of potassium near the membrane if the addition of the negative after-potentials were linear. Figure 15 demonstrates this; the initial height of the first negative after-potential is added to each negative after-potential during a train of impulses. It is therefore concluded that potassium ions extruded from the axon during excitation are retained for some time in

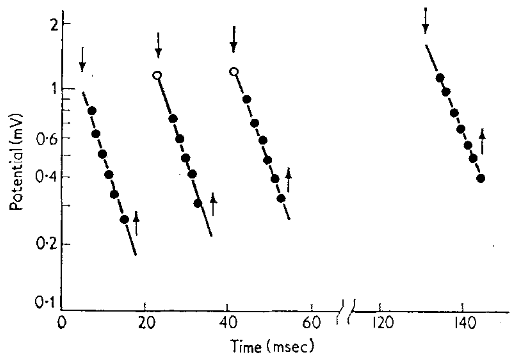

FIG. 15. Time course of the decay of the negative after-potential during a train of impulses of 50 c/s in the cockroach giant axon. The first, second, third and eighth impulses are illustrated. The ordinate represents the potential on a logarithmic scale, and the abscissa is the time measured from the beginning of the train. The filled circles are the measured points. The straight lines were drawn by eye. The open circles represent the potential immediately after the end of the second and third spikes, calculated by adding the initial height of the first negative after-potential to the after-potential remaining at the moment when the second and third spikes arise. The upward arrows indicate the start of the spikes, and the downward ones the end of the spikes (from Narahashi and Yamasaki, 1960a).

the immediate vicinity of the membrane, thereby producing a negative after-potential on depolarization. The increase in potassium concentration is estimated as about 2·0 mM.

g. Diffusion of potassium through the outer layer. The time constant of the falling phase of the negative after-potential is estimated as 9·2 msec, which is about twice as long as the membrane time constant of 4·2 msec (Yamasaki and Narahashi, 1959b). This means that the time constant of the negative after-potential is partly determined by the membrane time constant. It follows that changing the latter should affect the former.

The time constant of the negative after-potential is shortened by raising potassium concentration. In one case, for example, the time constant is estimated as 15·2 msec (0mM K), 8·9 msec (3·1 mM K) and 5·1 msec

(10 mM K). Since the membrane resistance is reduced by increasing the potassium concentration in other kinds of nerve and muscle (e.g. *Carcinus* axons, Hodgkin, 1947b; lobster axons, Narahashi, unpublished; frog muscle fibers, Tamasige, 1951; frog nerve fibers, Hashimura and Wright, 1958), it is reasonable to assume that the membrane time constant is reduced by high-K media in the cockroach giant axons. This should decrease the time constant of the negative after-potential as has actually been observed.

It is interesting to compare the time constant of the negative after-potential with that obtained with squid giant axons. The latter is estimated as 30–100 msec (Frankenhaeuser and Hodgkin, 1956), which is much longer than the membrane time constant of the squid axons of about 1 msec (Cole and Hodgkin, 1939; Hodgkin *et al.*, 1949). Therefore diffusion of potassium through the outer layer is much slower in squid than in cockroach axons. No attempt has been made to calculate the permeability of the outer barrier to potassium as has been done for squid giant axons (Frankenhaeuser and Hodgkin, 1956).

2. *Effect of calcium on after-potentials*

A rise in calcium concentration causes a marked increase in the negative after-potential (Narahashi and Yamasaki, 1960a). The positive phase disappears and is seen as a dip between the spike potential and the negative after-potential. Thus the configuration of the slow phase mimics that of the normal axon at high temperature, such as shown in Fig. 11. The falling phase of the negative after-potential is expressed as a single exponential term, the term constant being slightly longer than normal.

A lowering of calcium concentration causes a decrease in the positive phase and of the negative after-potential (Narahashi and Yamasaki, 1960a). The time constant of the falling phase of the negative after-potential is slightly shorter than normal.

There are three possible explanations for these changes in the after-potentials: (1) a change in resting potential; (2) a rise in potassium leakage per impulse; and (3) a decrease in potassium permeability immediately after the spike. As described in Section III, D, lc, the after-potentials are very sensitive to displacement of the membrane potential. Since the resting potential is raised by high-Ca, the first explanation explains, at least in part, the observed change in after-potentials. The second explanation above is based on the observation in the voltage-clamped squid axon that a rise in calcium causes potassium current to increase at depolarizations above some 40 mV (Shanes *et al.*, 1959). The third explanation is

based on the evidence from the squid axon that at small depolarizations the rise in potassium conductance is appreciably smaller when calcium concentration is raised (Frankenhaeuser and Hodgkin, 1957; Shanes *et al.*, 1959). However, it is at present impossible to determine to what extent explanations two and three are applicable to the present case.

3. *Effect of barium on after-potentials*

It is known that barium causes a remarkable prolongation of action potential (crustacean muscle fibers, Fatt and Ginsborg, 1958; mammalian B and C fibers, Greengard and Straub, 1959; grasshopper muscle fibers, Werman *et al.*, 1961; lobster muscle fibers, Werman and Grundfest, 1961). In Section III, C, 12, the effect of barium on the resting and spike potentials is described. Barium is also found to have a profound effect on the after-potentials (Narahashi, 1961a).

In Fig. 9 is shown the change in after-potentials produced by barium. The positive phase disappears and the negative after-potential is augmented. Unlike the normal negative after-potential, the large negative after-potentials in barium do not add on repetitive stimulation. This excludes the possibility that the negative after-potential in barium is caused by a large accumulation of some depolarizing substance such as potassium in the immediate vicinity of the membrane. In spite of large amplitude of the negative after-potential in barium, the time constant of the falling phase is relatively short, 4·7 msec on the average. This value is much shorter than the membrane time constant in barium, which is estimated as 14·0 msec, and is of the same order of magnitude as that measured for the falling phase of the catelectrotonic potential. This may imply that the mechanism accelerating the falling phase of the catelectrotonic potential is also operative during the falling phase of the negative after-potential. This is most probably due to the rise in potassium conductance. The membrane resistance is considerably increased by barium, though rectification is still present, suggesting that a small rise in potassium conductance still occurs upon depolarization (Fig. 6). Furthermore, it is shown in Section III, C, 12, that in barium solutions the sodium-carrying system is stabilized at a low level, suggesting that little inactivation occurs upon depolarization. These two factors, i.e. a partial suppression of the potassium conductance rise and of sodium inactivation, would cause a slowing of the falling phase of the action potential as has actually been observed, thereby producing a large negative after-potential. When the membrane potential reaches the initial height of the negative after-potential, the membrane is slowly repolarized, but the repolarization is slightly accelerated by the increased potassium conductance which is still higher than normal.

IV. Ionic Composition of Hemolymph and Nerve Activity

It is well known that in many kinds of animals sodium concentration is higher in the serum than in the axoplasm, while potassium concentration is higher in the axoplasm than in the serum. Thus, concentration gradients across the membrane are established with respect to sodium and potassium. For this reason, the excised nerve can maintain its excitability in a physiological solution which contains a large amount of sodium and small amounts of potassium and calcium. However, the situation is quite different in certain kinds of insects, whose hemolymph contains a large amount of potassium and a small amount of sodium. It seems difficult for the nerve to maintain normal excitability in such a solution. Although this problem has been subjected to physiological analyses for the past 20 years, the accumulated data are still too scant to draw any final conclusions. Despite this, some kinds of insects undoubtedly provide an excellent opportunity to elucidate the role of sodium and potassium in excitability. In the present article attention will mostly be focused on insect nerve, but some results on the insect muscle will be cited for comparison.

A. Ionic Composition of Hemolymph and Nerve

Ionic composition of the hemolymph has been estimated in a large number of species of insects (Asperen and Esch, 1956; Babers, 1938; Bishop *et al.*, 1925; Boné, 1944, 1946; Brecher, 1929; Clark and Craig, 1953; Drilhon, 1934; Duchâteau *et al.*, 1953; Hoyle, 1955; Ramsey, 1953; Tobias, 1948a, b; Treherne, 1961a). Some examples of Na and K contents are given in Table V. It will be seen that most carnivorous insects have a high Na/K ratio, whereas most herbivorous insects have a low Na/K ratio (Boné, 1944). The data on the ionic composition of the nervous system are very few (Table VI).

The ionic composition of the hemolymph is not stable but changes, depending on foods (Tobias, 1948a). For example, when fed on a leaf diet for 12–18 days, potassium in the serum of the cockroach is raised from 17·3 mM/l tissue water to 26·9 mM/l tissue water, while sodium is raised to a smaller extent, i.e. from 107 to 119 mM/l. When KCl solution is given orally, the increase in potassium concentration is more significant. In terms of Na/K ratio, the value is lowered from the control of 6·2, which is obtained with the standard mixed food, to 4·4 with leaf diet and to 2·7 with KCl administration. Under these conditions, however, no apparent symptoms of poisoning are produced. Thus, the nerve and muscle can

TABLE V

Sodium and potassium concentrations in hemolymph

Order	Species	Stage*	Food†	Na (mM/l)	K (mM/l)	Na/K	Reference
Coleoptera	*Dytiscus marginalis*	A	C	140	5	28·0	Ramsey (1953)
	,,	A	C	133	10	13·3	Boné (1944)
	Cicindela maritima	A	C	162	9	18·0	,,
	Tenebrio molitor	L	PP	86	45	1·9	,,
	,,	L	PP	66	43	1·5	Ramsey (1953)
	Melolontha vulgaris	A	H	6	49	0·12	Boné (1944)
Hemiptera	*Rhodnius prolixus*	A	C	158	5	31·6	Ramsey (1953)
	Triatoma megista	A	C	133	5	26·6	Boné (1944)
	Gerris najas	A	C	142	8	17·8	,,
	Cimex lectularius	A	C	139	9	15·5	,,
	Palomena prasina	A	H	22	42	0·52	,,
Diptera	*Tabanid* sp.	L	C	151	5	30·2	Ramsey (1953)
	Stomoxys calcitrans	A	C	128	11	11·7	Boné (1944)
	Dictenidia bimaculata	L	PP	39·6	3·7	10·7	Duchâteau, Florkin and Leclercq (1953)
	Tipula paludosa and oleracea	L	PP	84·8	8·2	10·3	,,
	Lucilia sericata	L	C	140	26	5·4	Boné (1944)
	Calliphora erythrocephala	L	C	148	37	4·3	,,
	Pegomyia sp.	L	H	26	58	0·45	,,

* L, Larva; P, Pupa; A, Adult.
† C, Carnivorous; H, Herbivorous; O, Omnivorous; PP, product of plants.

TABLE V—*cont.*

Order	Species	Stage	Food	Na (mM/l)	K (mM/l)	Na/K	Reference
Hymenoptera	*Vespula germanica*	L	O	48	41	1·2	Boné (1944)
	Apis mellifica	L	PP	10	45	0·22	,,
	Tenthredinid	L	H	6	55	0·11	,,
	Pteronidea ribesii	L	H	1·6	43·4	0·036	Duchâteau, Florkin and Leclercq (1953)
Lepidoptera	*Galleria mellonella*	L	O	26·5	36·3	0·73	Duchâteau, Florkin and Leclercq (1953)
	Prodenia eridania	L	H	22·3	39·7	0·56	,,
	Cossus cossus	L	PP	18·4	35·4	0·51	,,
	Vanessa urticae	L	H	22	43	0·51	Boné (1944)
	Bombyx mori	L	H	14	35	0·4	,,
	,,	L	H	13·0	37·2	0·34	Tobias (1948b)
	,,	L‡	H	3·4	41·8	0·081	Duchâteau, Florkin and Leclercq (1953)
	,,	L§	H	14·6	46·1	0·31	,,
	,,	P	—	11·3	41·5	0·27	,,
	,,	A	—	14·3	36·1	0·39	,,
	Phlogophora meticulosa	L	H	12·3	34·9	0·35	,,
	Ephestia kühniella	L	PP	17	60	0·28	Boné (1944)
	Pieris rapae	L	H	11	39	0·28	,,
	Pieris brassicae	L	H	7	28	0·25	Ramsey (1953)
	Cucullia absinthii	P	—	9·8	48·7	0·20	Duchâteau, Florkin and Leclercq (1953)
	Graellsia isabellae	P	—	6·2	46·2	0·13	,,
	Tropaea luna	P	—	4·4	52·8	0·083	,,

‡ Third instar. § Fifth instar.

TABLE V—*cont.*

Order	Species	Stage	Food	Na (mM/l)	K (mM/l)	Na/K	Reference
Orthoptera	*Gryllotalpa gryllotalpa*	A	O	233·7	7·3	32·0	Duchâteau, Florkin and Leclercq (1953)
	Periplaneta americana	A	O	106·0	24·1	4·3	Tobias (1948a)
	,,	A	O	156·0	7·7	20·2	Asperen and Esch (1956)
	,,	A	O	157·4	12·3	12·7	Treherne (1961a)
	Gryllotalpa vulgaris	A	O	174	11	15·8	Boné (1944)
	Schistocerca gregaria	L	H	81·3	5·3	15·3	Duchâteau, Florkin and Leclercq (1953)
	Locusta migratoria migratoriodes	A	H	109	18	6·0	Hoyle (1954a)
	,,	L	H	60	12	5·0	Duchâteau, Florkin and Leclercq (1953)
	,,	A	H	83	19	4·3	Ramsey (1953)
	Romalea microptera	A	H	56·3	15·8	3·5	Tobias (1948b)
	Locusta viridissima	A	O	83	51	1·6	Boné (1944)
	Stenobothrus stigmaticus	A	H	61	62	0·98	,,
	Dixippus morosus	A	H	14	16	0·87	Ramsey (1953)
	Carausius morosus	A	H	21	25	0·84	Boné (1944)
Trichoptera	*Phryganea* sp.	L	PP	92·0	6·8	13·5	Duchâteau, Florkin and Leclercq (1953)
	Chaetopteryx villosa	L	H	63	9	7·0	Boné (1944)

TABLE VI

Ionic composition of nerves and muscles

Animal	Tissue	Na	K	Mg	Unit	Reference
Periplaneta americana	Nerve cord	83·9	140	4·0	mM/l tissue water	Tobias (1948a)
,,	Muscle	45·6	112	7·4	,,	,,
,,	Nerve cord	103·2	180·2		mM/l	Treherne (1961a)
,,	Abdominal ganglion	110·5			mM/l tissue water	Treherne (1961c)
Romalea microptera	Nerve cord	89	55		mM/l tissue water	Tobias (1948b)
,,	Muscle	44	128		,,	,,
Squid (*Loligo*)	Axoplasm of giant axon	44	369		mM/kg	Steinbach and Spiegelman (1943)
,,	,,	46	323		,,	Keynes and Lewis (1951)
,,	,,	65	344	20	,,	Koechlin (1955)
Human	Brain	81·8	93·6		mM/l tissue water	Shohl (1939)
,,	Muscle	39·2	117·9		,,	,,

tolerate a relatively wide variation of the external potassium concentration. A similar result has been obtained for the grasshopper, *Locusta migratoria migratorioides* (Hoyle, 1954a). When the adult is starved, the sodium and potassium concentrations in the hemolymph are estimated as 103 and 11 mM/l respectively as against the control values of 109 and 18 mM/l, which are obtained from the adult fed on grass. However, in the 5th instar nymph, the sodium concentration is increased by starvation (from 91 to 109 mM/l) while the potassium concentration is greatly reduced (from 30 to 14 mM/l).

B. EFFECT OF EXTERNAL CONCENTRATIONS OF CATIONS ON NERVE AND MUSCLE

1. *Silkworm nerve cord*

The hemolymph of the silkworm contains a large amount of potassium; sodium concentration is low (Boné, 1944; Duchâteau *et al.*, 1953; Tobias, 1948b). An attempt was made to find a physiological solution in which the spontaneous activity of the central nerve cord was maintained as long as possible (Narahashi, unpublished). Some thirty kinds of physiological solution were prepared, the ratios of Na, K, Ca and Mg being altered. Some of them contained a large amount of Na and small amounts of K, Ca and Mg, while others contained a large amount of K, more closely mimicking the hemolymph. It was found that solutions in which Na, K, Ca and Mg are at about the same concentrations as in the hemolymph suppress spontaneous activity rather quickly. On the other hand, solutions whose ionic composition mimics that of the ordinary physiological solution for frog and mammals can maintain spontaneous activity longest. The best solution in this sense has the following composition (mM): Na^+, 150; K^+, 3; Ca^{2+}, 3; pH adjusted at 6·6–6·8 by phosphate buffer. During the course of this experiment, it was also found that potassium and calcium antagonize each other; the effect of increasing K can be partly suppressed by increasing Ca. Further, magnesium has an action similar to calcium, but is less potent.

There are at least three possible explanations for these results: (1) The experiments were done with the isolated nerve cord preparation. It seems probable that the physiological conditions of nerve fibers are changed in some way by dissection. In other words, the nerve cord may become sensitive to the external composition by isolation. (2) It is possible, though unlikely, that potassium in the hemolymph exists in a bound form. In the cockroach, excellent agreement is obtained between the high-K level normally found to be tolerated *in vivo* and that found to be tolerated by

the excised nerve cord in Ringer's solution, excluding the possibility of the hemolymph potassium existing as a bound form (Tobias, 1948a). However, since no such data are available for the silkworm larvae, it cannot be decided whether potassium exists in a bound form in the silkworm hemolymph. (3) There is evidence in other insects that the nerve sheath surrounding the nerve cord acts as an effective barrier to the diffusion of ions as will be described later (IV, B, 2 and 3). However, even if the barrier did exist around the nerve cord of the silkworm, this could not explain the result unless one assumed a change in ionic permeability of the sheath upon isolation.

In the locust nerve, when oxygen supply through the tracheal system is suspended, the nerve sheath becomes less effective as a diffusion barrier (Hoyle, 1953). A partial oxygen deficit may have occurred under the present experimental conditions, because the nerve cord was completely isolated from the silkworm larva. Furthermore, experiments on the movements of sodium and potassium in the nerve cord of the cockroach have shown that a dynamic steady-state rather than a static impermeability exists across the sheath, suggesting close linkage to metabolism (Treherne, 1961a). It is therefore reasonable to assume that the nerve sheath loses by isolation its ability to maintain a dynamic steady-state, thereby making the nerve cord sensitive to changes in external ionic concentrations.

All of the three possibilities remain speculative at present. Since this experiment was made some 10 years ago and left unrepeated since then, it is necessary to explore this problem by improved electrophysiological techniques and to examine ionic fluxes through the nerve sheath in order to draw final conclusions.

2. *Cockroach nerve*

In the hemolymph of the cockroach the sodium concentration is higher than the potassium, resembling the serum of higher animals (Asperen and Esch, 1956; Treherne, 1961a; Tobias, 1948a). The sheath surrounding the nerve cord has been demonstrated to act as a strong diffusion barrier, suggesting the possibility that the external fluid which is actually in contact with the nerve fibers may be different in ionic composition from the hemolymph. When an intact sheathed segment of the nerve cord is bathed in 180 mM KCl, it takes 12–18 min for conduction to be blocked, whereas it takes only 10 sec for conduction to be blocked if the desheathed segment is treated in the same way (Twarog and Roeder, 1956). This is also the case in Na-deficient saline; conduction is not impaired for several hours when the sheathed region is treated, whereas blockage occurs within 30 sec when the desheathed region is treated.

These results were confirmed by the author. The sheath also acts as a barrier against the penetration of acetylcholine; acetylcholine becomes effective in blocking synaptic transmission across the last abdominal ganglion if the sheath is removed (Twarog and Roeder, 1956, 1957; Yamasaki and Narahashi, 1960). In general, compounds in ionized form penetrated through the barrier with difficulty, but unionized substances penetrate more readily (O'Brien, 1957). This explains the non-toxicity of atropine, prostigmine, and acetylcholine and its analogues, and the low toxicity of eserine.

The barrier may act as a buffer to maintain the ionic composition actually surrounding the nerve fibers constant. When the cockroach is fed on a leaf diet or given KCl solution, the ratio K cord/K serum is decreased from the control value of 8·1 to 6·6 in the former case, and to 4·5 in the latter (Tobias, 1948a). The decrease in the ratio means that the change in potassium concentration is more marked in the serum than in the nerve cord in terms of percentage.

The apparent impermeability of the nerve sheath to ions as demonstrated by electrophysiological methods does not necessarily mean that the nerve sheath does not permit ions to pass through it. In other words, a dynamic steady-state rather than a static impermeability can exist across the nerve sheath. This has actually been demonstrated by isotope experiments (Treherne, 1961a). This problem in the cockroach nerve will be discussed in greater detail in Section V.

The sheath of the cockroach nerve cord is composed of two layers, i.e. an outer homogeneous, non-cellular layer called neural lamella, and an inner cellular layer called perineurium (cf. Section II). The two layers are closely adherent to one another and pulled away together from the nerve substance when the preparation is desheathed (Twarog and Roeder, 1956). It is suggested that it is the inner cellular perineurium which plays an important role as a diffusion barrier (Treherne, 1961a; Twarog and Roeder, 1956; Wigglesworth, 1960).

3. *Grasshopper nerve and muscle*

The hemolymph of the grasshopper, *Locusta migratoria migratorioides*, contains 109 mM Na and 18 mM K. Therefore the ratio Na/K is the same direction as is that of higher animals. As in the case of the cockroach, the sheath surrounding the nerve has been shown to act as a strong barrier against the penetration of ions (Hoyle, 1952, 1953). When 70 mM K is applied to the intact nerve, conduction is not impaired over several hours; 140 mM K blocks the conduction after 2–3 h. However, when injected under the sheath, potassium in a concentration as low as 40 mM causes

a very rapid block. Unlike neural tissues, muscle fibers are not surrounded by a sheath. The muscle fiber membrane is depolarized by high-K, the resting potential being linearly related to the logarithm of the external potassium concentration at the higher concentrations (Hoyle, 1953). These results mean that although the nerve fibers are protected to some extent from the immediate change in potassium concentration in the hemolymph, the resting potential of the muscle fibers is affected quickly by the change in potassium concentration. Since the feeding condition affects the hemolymph potassium, neuromuscular activity is partly determined by the feeding condition, and the direction of the change is such that starvation enhances neuromuscular activity (Hoyle, 1953, 1954a).

In the case of the grasshopper nerve also, a dynamic steady-state rather than static impermeability can be expected to exist across the nerve sheath (Treherne, 1961a), because the ability of the nerve sheath to prevent external ions from penetrating is suppressed by suspending the oxygen supply (Hoyle, 1953).

4. *Physiological solutions*

Many kinds of physiological solutions have so far been used for insects. Table VII gives some examples. It is usually found that insect nerves are rather insensitive to small changes in ionic composition. For this reason a physiological solution which had been used for a certain kind of insect could also be used for other insects without modifying its composition. However, whenever the exact value for the normal membrane potential is required and the experiment involves a long-term observation, the composition should be carefully determined. The author has recently been using a modified physiological solution which contains a greater amount of sodium for the cockroach giant axons (Yamasaki and Narahashi, 1959a). When the old solution which contains 159·6 mM Na is used, there is a tendency for the axons to swell. This is because the hemolymph of the cockroach is isotonic to 224 mM NaCl (Ludwig *et al.*, 1957). The swelling is not seen when the sodium concentration is raised to 214 mM.

V. IONIC FLUXES AND METABOLISM

It has been established in the giant axons of the squid that there is a mechanism by which intracellular sodium is pumped out, keeping the sodium concentration smaller in the axoplasm than in the external fluid, and that energy is supplied by metabolism to operate this mechanism (cf. Hodgkin, 1958). Electrophysiological experiments with the cockroach giant axons have demonstrated that sodium and potassium ions play

TABLE VII

Physiological solutions for insects

Animal	Tissue	Na (mM)	K (mM)	Ca (mM)	Mg (mM)	Buffer	Glucose (mM)	pH	Reference
Periplaneta americana	Nerve, muscle	153·8	2·7	1·8			22		Pringle (1938)
,,	,,	153·8	2·7	1·8		*	22	7·2	Roeder (1948b)
,,	,,	130	10	2	2	$H_2PO_4^-$, HCO_3^-			Twarog and Roeder (1956)
,,	,,	154	2·6	1·9		HCO_3^-	22		Boistel (1960)
,,	,,	159·6	3·1	1·8		$H_2PO_4^-$, HPO_4^{2-}		7·2	Yamasaki and Narahashi (1959a)
,,	,,	214	3·1	1·8		$H_2PO_4^-$, HPO_4^{2-}		7·2	,,
,,	Heart	186·8	21	7·6	1·7				Yeager (1939)
,,	,,	209·7	12·8	5·5		$H_2PO_4^-$, HCO_3^-		7·6–7·8	Yeager (1938)
,,	,,	200†	20†	15†					Ludwig, Tracey and Burns (1957)
,,	Nerve	157	12·3	4·5	4·0	$H_2PO_4^-$, HCO_3^-	2·2‡		Treherne (1961a)
,,	Nerve, muscle	171	3	2		HPO_4^{2-}		7·2	Welsh and Gordon(1947)

* Both phosphate and bicarbonate are tried.
† The composition in which the heart beat is kept longest.
‡ In addition to glucose, trehalose 36·9 mM, glycine 30·0 mM, glutamic acid 35·0 mM, and glutamine 30·0 mM are contained.

TABLE VII—*cont.*

Animal	Tissue	Na (mM)	K (mM)	Ca (mM)	Mg (mM)	Buffer	Glucose (mM)	pH	Reference
Locusta migratoria migratorioides	Nerve, muscle	140	10	2	2	$H_2PO_4^-$, HCO_3^-			Hoyle (1953)
Locusta migratoria danica	Heart	168	10·3	4·5		$H_2PO_4^-$, HCO_3^-		7·5–8·0	Yamasaki and Ishii (1950a)
Allomyrina dichotomus	Nerve	168	10·3	4·5		$H_2PO_4^-$, HCO_3^-		6·6–6·8	Yamasaki and Ishii (1952a)
,,	Heart	168	10·3	4·5		$H_2PO_4^-$, HCO_3^-	5·5	7·5–8·0	Yamasaki and Ishii (1950b)
Anax imperator	Nerve	130	1·3	1·8		HCO_3^-			Fielden (1960)
Bombyx mori	Nerve, heart	150	3	3		$H_2PO_4^-$, HPO_4^{2-}		6·6–6·8	Narahashi (unpublished)
Musca domestica (Larva)	Heart	146	4·8	1·5					Brebbia and Ludwig (1962)
Musca domestica (Pupa)	,,	219·4	4·4	1·1		$H_2PO_4^-$, HPO_4^{2-}	14	7·0	,,
Musca domestica (Adult)	Heart	246·3	4·8	1·6		$H_2PO_4^-$, HPO_4^{2-}	14	7·0	Brebbia and Ludwig (1962)
Musca domestica vicina	Nerve, muscle	214	3·1	1·8		$H_2PO_4^-$, HPO_4^{2-}		7·2–7·4	Yamasaki and Narahashi (1958a)

roles similar to those in the squid giant axons in maintaining membrane potential and excitability (Section III). It would then be expected that the so-called "sodium-pump" may also exist in the nervous system of the cockroach. This has been demonstrated in a series of isotope experiments performed by Treherne (1961a–e, 1962).

As has been discussed in a preceding section (IV), there is evidence that the sheath surrounding the nerve cord acts as a barrier to the penetration of sodium, potassium and certain kinds of drug. On the contrary, experiments on the flux of glucose and trehalose in the cockroach nerve have shown that there is a rather rapid influx of such large molecules despite the presence of the nerve sheath (Treherne, 1960). This raises a question about the nature of the nerve sheath as a diffusion barrier. Experiments by the use of isotopes have resulted in an apparently paradoxical situation, sodium and potassium ions being able to pass through the nerve sheath very quickly (Treherne, 1961e) as will be discussed below.

A. INFLUX OF SODIUM AND POTASSIUM INTO NERVE

The influxes of sodium and potassium into the nerve cord of the cockroach have been measured after injection of ^{24}Na and ^{42}K into the hemolymph (Treherne, 1961a). The specific activities of ^{24}Na and ^{42}K in the nerve cord increase with time after the injection, and finally attain the same level as that in the hemolymph. The influxes of these ions can be calculated using an equation describing the exchange of ions between the isolated living cells and the surrounding media:

$$m_i = -\frac{1}{t} C_i \log_e \left(1 - \frac{A_i}{A_o}\right) \tag{14}$$

where m_i is the inward ionic flux, t is time, C_i is the concentration of ion in the nerve cord, A_i and A_o are the specific activities in the nerve cord and the hemolymph respectively. The influxes of sodium and potassium are calculated as 320 mM/l of nerve cord water/h and 312 mM/l of nerve cord water/h respectively. The influx per unit area of nerve cord surface can be calculated as: Na, $13{\cdot}9 \times 10^{-12}$ M cm^{-2} sec^{-1}; K, $13{\cdot}5 \times 10^{-12}$ M cm^{-2} sec^{-1}.

The influx of potassium ions is comparable to the value of $16{\cdot}7 \times 10^{-12}$ M cm^{-2} sec^{-1} obtained for *Sepia* axons (Keynes, 1951). On the contrary, the influx of sodium ions is much smaller than that of $61{\cdot}0 \times 10^{-12}$ M cm^{-2} sec^{-1} obtained for *Sepia* axons. However, since the sodium concentration in the external medium is much higher in the experiment with *Sepia* than in that with the cockroach, the difference in the sodium influx may in fact be much smaller.

These values for ionic fluxes need re-examination (Treherne, 1961d) since the assumptions used in calculation are over-simplified. It has been assumed that the rate-limiting process is the transfer across the superficial boundary and that the movements within the underlying layers occur rapidly so that the ions are well-mixed, but the actual situation is probably more complicated.

In spite of this inaccuracy, the rapid flux of ions is rather surprising in view of the electrophysiological experiments which show the sheath to be an efficient barrier to the penetration of ions (Hoyle, 1952, 1953; Twarog and Roeder, 1956, 1957; Yamasaki and Narahashi, 1959a, 1960). It is suggested that a dynamic steady-state rather than a static impermeability must exist across the nerve sheath (Treherne, 1961a). This suggestion is supported by the observation that anoxia reduces the efficiency of the sheath as a diffusion barrier (Hoyle, 1953), and that the sodium efflux is metabolism-dependent in nature (Treherne, 1961b, c). However, as will be discussed later (V, C), this explanation is not satisfactory; the diffusion of ions from the extracellular space to the external medium was found to be very rapid and to be unaffected by the metabolic inhibitor, 2:4-dinitrophenol (Treherne, 1961e).

B. EFFLUX OF SODIUM

1. *The nerve cord of the cockroach*

The efflux of radioactive sodium from the nerve cord of the cockroach has been measured under various conditions (Treherne, 1961b, c). There is an initial exponential decline in radioactivity which at a certain point is abruptly followed by a second slower exponential phase (Fig. 16). The

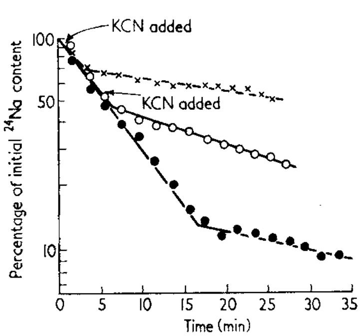

FIG. 16. The effect of 10·0 mM KCN on sodium efflux. The cockroach nerve cord (from Treherne, 1961c).

initial rapid phase is effectively slowed by potassium cyanide or 2:4-dinitrophenol (Fig. 16). The same effect is observed when potassium is removed from the medium (Fig. 17). On the contrary, the replacement of sodium with choline or xylose has little or no effect on the rate of sodium efflux.

The ability of metabolic inhibitors to suppress the rate of sodium efflux implies that the latter is linked in some way with metabolism, in agreement with results obtained with squid giant axons (Hodgkin and Keynes, 1955). It can be seen in Fig. 16 that under the influence of a metabolic inhibitor the rate of sodium efflux from the cockroach nerve

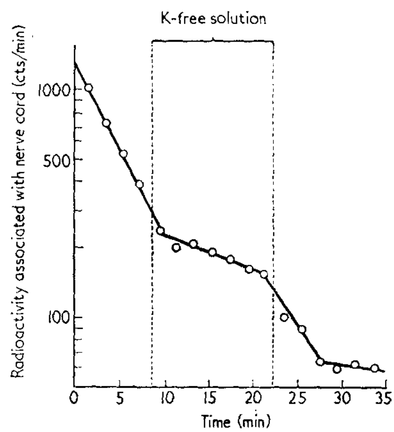

FIG. 17. The effect of potassium-free solution on ^{24}Na efflux from the isolated abdominal nerve cord of the cockroach (from Treherne, 1961c).

cord approaches that of the slow phase in normal solution. It is therefore suggested that the slow phase represents some sort of breakdown of the normal extrusion mechanism in the isolated nerve cord. The suppression of sodium efflux by K-free media suggests coupling between sodium efflux and potassium influx, evidence for which has been obtained in other tissues (squid giant axons, Hodgkin, 1958; Hodgkin and Keynes, 1955; muscle, Keynes, 1954; Steinbach, 1952; red cells, Glynn, 1956; Harris and Maizels, 1951; frog skin, Ussing, 1954).

2. *The last abdominal ganglion of the cockroach*

The efflux of sodium has also been measured using the last abdominal ganglion of the cockroach (Treherne, 1961d). One finds a single exponential phase of decline not followed by a slow phase. The important obser-

vation is that removal of the sheath has little, if any, effect on the efflux rate (Fig. 18). In accordance with the nerve cord experiment, 2:4-dinitrophenol and K-free media slow the sodium efflux.

The lack of effect of desheathing on the rate of sodium efflux leads us to conclude that the rate-limiting process in the efflux of sodium is not the transfer of ions across the nerve sheath. It then follows that the transfer of sodium ions through the nerve sheath occurs very rapidly. The rate of sodium efflux is mostly determined by the transfer of sodium ions across the cell membrane of the underlying tissue.

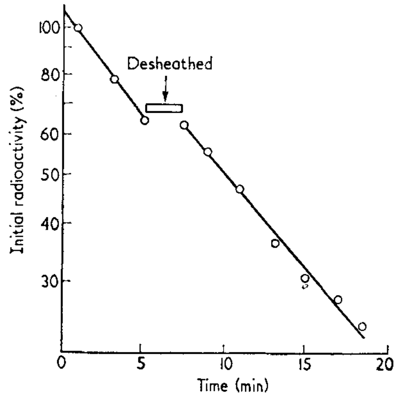

FIG. 18. The effect of removing the connective tissue and cellular sheath from the dorsal surface of the ganglion of the cockroach on the rate of loss of ^{24}Na when washed in inactive solution (from Treherne, 1961d).

3. *Rates of diffusion of sodium from the cellular components and from the extracellular space*

By measuring the rapid initial component of sodium efflux, it was possible to distinguish the rate of diffusion from the cellular components from that from the extracellular space (Treherne, 1961e). Figure 19 shows the initial part of the sodium efflux. It will be seen that the efflux occurs rapidly with a complex time course, and then follows a slow exponential time course. By subtracting the slow component, which can be extrapolated to zero time, a pure fast component can be drawn. The fast component is complex initially, but after a few seconds it assumes a simple exponential form with a half-time of about 33 sec. The half-time for the slow component is about 260 sec. It should be remembered that the slow phase observed here corresponds to the initial rapid phase in the former experiments (Fig. 16) in which the very early phase of sodium efflux cannot be measured.

The presence of 2:4-dinitrophenol does not affect the fast component of sodium efflux, but the slow component is effectively reduced in rate. Under the influence of 0·5 mM 2:4-dinitrophenol, the mean value for the fast component is estimated as 31·5 sec and the normal mean is estimated as 28·4 sec. The mean value for the slow component in 2:4-dinitrophenol is 593 sec, as against the normal mean value of 277 sec. This result agrees well with the former observation that 2:4-dinitrophenol inhibits the sodium efflux (Fig. 16).

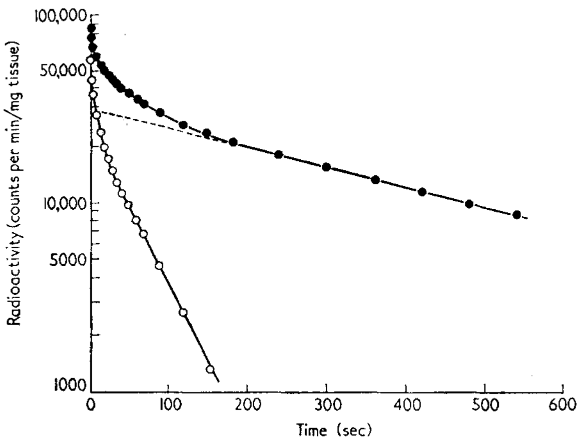

FIG. 19. The loss of ^{24}Na from a cockroach nerve cord, loaded for 10 min, when washed in inactive solution (●). The fast component of the main curve (○) was obtained by subtraction from the straight line extrapolated to zero time (from Treherne, 1961e).

The diffusion constant of sodium in the extracellular space can be calculated by the following equation (Hill, 1928):

$$t_{0\cdot5} = 0\cdot118 r_o^2/D', \tag{15}$$

where $t_{0\cdot5}$ is the half-time, r_o is the radius of the connective, and D' is the diffusion constant in the extracellular space. Taking the half-time of 43 sec and assuming the radius of the connective as 0·145 mm, the diffusion constant in the extracellular space is calculated to be $5\cdot77 \times 10^{-7}$ cm^{-2} sec^{-1}, which is about one-thirtieth of that for the free diffusion of sodium ions. The reduction in apparent diffusion constant suggests an increase in the effective path length for ions diffusing between a complex collection of cellular structures. Furthermore, the removal of the sheath has little effect on the slow component of the sodium efflux. These results lead us to the hypothesis that the fast component of the sodium efflux represents the movements of ions in the extracellular spaces.

The experiments with ^{14}C-inulin support this view. The extracellular spaces are determined as 10% by the use of ^{14}C-inulin. The ratio of the half-time for sodium efflux to that for inulin escape is of the same order of magnitude as the reciprocal of the ratio of the free diffusion constant for inulin to that for sodium. This means that the fast component in sodium efflux is a movement within the extracellular space as demonstrated by ^{14}C-inulin.

It is then reasonable to assume that the slow component of the sodium efflux, which corresponds to the initial rapid phase in the earlier experiments (Fig. 16), represents an extrusion from the cellular components of the nervous system. Thus, this component reflects activity of the sodium-pump as studied in squid giant axons (cf. Hodgkin, 1958). It is inhibited by metabolic inhibitors, suggesting the importance of metabolic energy in maintaining its activity. It is also inhibited by removal of potassium from the external fluid, suggesting a linkage between the sodium extrusion and potassium uptake.

4. *Nature of the nerve sheath*

In view of the fast transfer of ions across the nerve sheath as discussed in the preceding section (V, B, 3), an apparent paradoxical situation concerning the role of the nerve sheath as a diffusion barrier requires a search for its explanation in other ways. It has been demonstrated that the levels of the various ions in the intercellular fluid are very different from those in the external medium, the cations being more and the chloride ions less concentrated than those in the external medium (Treherne, 1962). Further, it is suggested that these ions distribute themselves between the intercellular spaces and the external medium according to a Donnan equilibrium. It follows that desheathing causes a fall of cation concentrations and a rise of anion concentrations in the solution directly surrounding the nerve fibers. It is probable that such changes in ionic environment may affect the rate of depolarization by high-K media or other media. Thus the well-known effect of desheathing in accelerating the block by high-K and low-Na may simply be ascribed to changes in ionic environment rather than to the removal of a diffusion barrier. However, this view must remain speculative until more detailed data become available.

C. EFFECT OF METABOLIC INHIBITION ON RESTING POTENTIAL AND EXCITABILITY

There is general agreement that the membrane potential is in some way supported by metabolic energy. Although the membrane potential reflects concentration gradients of ions across the membrane and selective

permeability of the membrane, the energy which enables ions to be distributed with concentration gradients and makes the membrane semipermeable to ions is thought to be supplied by metabolism. It is hoped that experiments with metabolic inhibitors will throw some light on the metabolic aspect of membrane potential production. The experiments which will be discussed below were performed by means of external electrodes some 10 years ago, so that it seems necessary to repeat the experiment with the aid of micro-electrodes which make the interpretation of the results more clear-cut (Yamasaki and Narahashi, 1957a).

Two fluid electrodes were in contact with the excised abdominal nerve cord of the cockroaches, *Periplaneta americana* and *P. suliginosa*, one at the central part of the nerve cord and the other at the end which was dipped into the isotonic KCl solution. Thus the so-called potassium potential was measured.

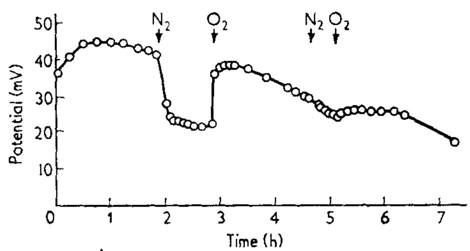

FIG. 20. Anoxic depolarization of the cockroach nerve cord (from Yamasaki and Narahashi, 1957a).

1. *Effect of oxygen lack on resting potential*

The resting potential measured by external electrodes amounts to about 40 mV. This value is about half of the absolute resting potential which is estimated as 77 mV on an average (Narahashi and Yamasaki, 1960a). The effect of oxygen lack on the resting potential is shown in Fig. 20. The resting potential gradually declines under this condition. Admitting nitrogen into the nerve chamber causes a fall of resting potential. The fall is rapid initially, followed by a slower decline, the potential finally attaining a low steady-state level which is about half of the normal resting potential. Readmitting oxygen brings about recovery of the resting potential, and on some occasions produces an initial overshoot. However, when nitrogen is again introduced into the chamber at a later time in the experiment when the resting potential has decreased to some extent, the fall of resting potential is much smaller than that at the first introduction of nitrogen. Further, the steady-state resting potential during the second

period of anoxia attains almost the same level as that during the first. In other words, an anoxia insensitive fraction of the resting potential remains unchanged during long-term measurement.

It is then concluded that the resting potential can be divided into two fractions: an anoxia sensitive fraction which is called "aerobic resting potential", and an anoxia insensitive fraction which is called "anaerobic resting potential". The anaerobic resting potential is stable and remains unchanged over many hours under these experimental conditions, while the aerobic resting potential is labile and reduced progressively during experiment. Each fraction comprises about 50% of the total resting potential.

2. *Effect of metabolic inhibitors on resting potential*

Monoiodoacetate (IAA) causes depolarization with a certain delay. At low concentrations of 10^{-4} M or 10^{-3} M, the fall of resting potential is attributed largely to the depression of the aerobic fraction of the resting potential, whereas at a high concentration of 2×10^{-3} M, the aerobic resting potential is completely depressed and the anaerobic one is also partly depressed.

Fluoride at a concentration of $2–3 \times 10^{-2}$ M also causes depolarization. Unlike IAA, the depolarization begins shortly after application of fluoride. The aerobic and anaerobic fractions of the resting potential are depressed to about the same extent.

Upon application of weak concentrations of cyanide (5×10^{-5} M–$2{\cdot}5 \times 10^{-3}$ M), the resting potential is decreased rapidly. However, there is a tendency for the resting potential to recover in the presence of cyanide, an overshoot being observed in some cases.

Some observations were made with K-rich solution for comparison. 15mM K solution causes depolarization, the aerobic and anaerobic fractions being depressed to the same extent.

3. *Excitability under the influence of metabolic inhibitors*

Under the influence of IAA and cyanide, depolarization is accompanied by conduction block. In the case of fluoride, however, conduction was not impaired with the concentrations used despite a depolarization larger than that produced by IAA and cyanide (Yamasaki and Narahashi, 1957d).

After conduction is blocked by IAA, cyanide or high-K, anodal hyperpolarization is effective in restoring excitability. Action potentials are elicited on stimulation during the passage of a prolonged anodal current. It takes some seconds for excitability to be restored by anodal current.

Anodal restoration is observed in a wide variety of experimental conditions in other kinds of nerves and muscles (e.g. Lorente de Nó, 1947a, b; Woronzow, 1924; Weidmann, 1955b; Koketsu and Noda, 1960; Posternak and Arnold, 1954; Schoepfle, 1957; Takeuchi and Tasaki, 1942; Narahashi, unpublished).

It should be kept in mind in interpreting these results with metabolic inhibitors that the depolarization observed may not be due to direct action of the drugs, but to the accumulation of potassium around the nerve membrane. The latter may occur because the ability of the nerve fibers to retain potassium is suppressed by metabolic inhibition. Thus, it has been found that washing the nerve under the same inhibitory conditions restores excitability (Feng *et al.*, 1950; Shanes, 1951a).

VI. Effect of Insecticides on Nerve Activity

Since the discovery of DDT as an insecticide, the neurotoxicology of insects has become a subject of increasing interest. This is because the knowledge of the mode of action of insecticides may provide excellent approaches to the improvement and development of insecticides. However, it is not the author's intention to cover the whole area of insect neurotoxicology in the present article. Instead, the mechanism of action of insecticides on insect nerve will be reviewed from the standpoint of neurophysiology. In other words, the insecticides used in the studies cited are regarded as tools rather than subjects. For this reason, some studies are not critically important from this point of view, and may be omitted in spite of their important contributions to insect toxicology.

A. DDT

1. *Outline of the effect of DDT on nerve*

DDT has long been known to stimulate the nerve to produce repetitive discharge. The sensory nerve of the cockroach is very sensitive to DDT, and produces spontaneous trains of impulses (Dresden, 1949; Lalonde and Brown, 1954; Roeder and Weiant, 1946, 1948; Yamasaki and Ishii, 1953, 1954a, b, 1957a–c). However, the exact site of origin of the impulses remains to be seen. Repetitive response is elicited in squid giant axons, crab axons and frog nerves when they are treated with DDT (Shanes, 1949a, b, 1951b). The repetitive response to DDT has been analysed in relation to the action of calcium (Gordon and Welsh, 1948; Welsh and Gordon, 1947). Repetitive responses elicited from the ganglion by direct current stimulation are found to be effectively augmented by treatment

with DDT (Yamasaki and Ishii, 1954c, 1957d). Meanwhile, another aspect of DDT action has been found during the course of observation on the spontaneous discharge of the cockroach cord treated with DDT (Yamasaki and Ishii, 1952b). The action potential is greatly prolonged. This effect of DDT was further studied (Yamasaki and Narahashi, 1957b) and was subjected to micro-electrode analysis (Yamasaki and Narahashi, 1957c; Narahashi and Yamasaki, 1960b, c). Thus there are at least two aspects of DDT action on the nerve, i.e. repetitive responsiveness and prolongation of the action potential. The latter is ascribed to the increase in negative after-potential. These two effects are related to each other as will be described below.

2. *Repetitive response*

DDT causes the nerve fibers to produce repetitive discharge in response to a single stimulus (Gordon and Welsh, 1948; Welsh and Gordon, 1947). Although most of the experiments of these workers were made with isolated nerves and with the nerve-muscle preparation of the crayfish, *Cambarus virilis*, and the crab, *Cancer irroratus*, the cockroach leg preparation yielded a similar result. A similar effect is also observed with other insecticides including pyrethrins, *p*-dichlorbenzen, naphthalene and nicotine, and with many DDT- analogues including difluoro analogue, dibromo analogue and dimethoxy analogue. Lowering calcium concentrations intensified the repetitive discharge under the influence of DDT, while raising it counteracted repetitiveness. Magnesium ions have an action similar to Ca, but are less potent.

Based on these observations, a hypothesis is put forward concerning the role of calcium in excitation and the mode of action of DDT on the nerve (Gordon and Welsh, 1948). Polar groups, possibly phosphoryl, are fixed in the axon surface at intervals, and a fraction of the available pairs are weakly bridged by calcium ions. Impulses may break some or all of the cross-linkages, but they are quickly reformed. If the restoration of calcium ions to a surface complex is delayed, repetitive firing occurs. Toxic molecules may interfere with surface re-calcification by disordering the axon surface structure. Although this hypothesis seems tempting, it will be discussed later that recent observations on DDT action made with micro-electrodes do not completely fit the hypothesis (Narahashi and Yamasaki, 1960b).

In the giant axons of the abdominal nerve cord of the cockroach, there is only a brief period during which repetitive discharge is elicited under the influence of DDT (Narahashi and Yamasaki, 1960b). When the negative after-potential is increased to a certain amount after treatment

with DDT, a condition is suddenly established under which a single stimulus produces repetitive firing (Fig. 21). The repetitive discharge lasts only for 30–70 msec. After some time, however, the condition of repetitive discharge suddenly vanishes, and a single stimulus can no longer elicit

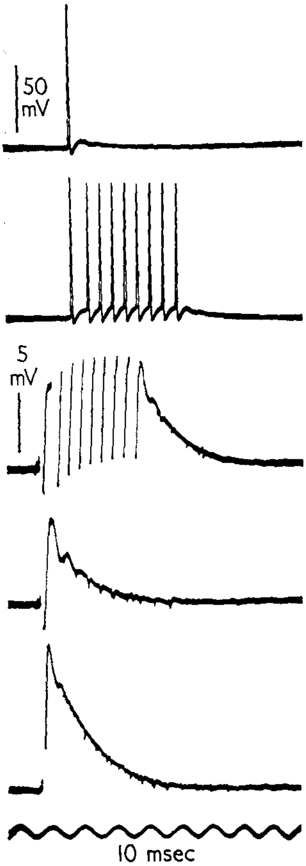

FIG. 21. Records of the action potential induced by a single stimulus before, during and after the repetitive stage in the course of DDT poisoning in the cockroach giant axon. From top to bottom, 20, 22, 26, 26·5 and 34 min after treatment with 10^{-4} M DDT (from Narahashi and Yamasaki, 1960b).

multiple discharge. The slightly augmented negative after-potential is no doubt one of the causes of repetitive excitation, but the involvement of other mechanisms is suggested by the fact that in normal axons a prolonged cathodal polarization does not produce repetitive firing even when

the current strength is raised beyond threshold. Therefore, the condition of the axon is changed by DDT in such a manner that a prolonged depolarization causes multiple response.

It should be noted that immediately after the subsidence of repetitive discharge in DDT solution there can be seen oscillatory potentials following the first impulse (Fig. 21). The oscillatory potentials are superimposed on a large negative after-potential of the first action potential, and damp progressively towards the end of the negative after-potential. The oscillatory property of the membrane is known to depend on the apparent inductance, resistance and capacity of the membrane (Cole and Curtis, 1941; Hodgkin and Rushton, 1946). The damped oscillations observed during a flow of cathodal current in *Carcinus* axons are attributed to a decrease in membrane resistance upon depolarization (Hodgkin and Rushton, 1946). It seems, therefore, probable that these membrane constants are changed by DDT in such a way as to favor oscillations.

According to the hypothesis put forward by Welsh and Gordon (1947) and Gordon and Welsh (1948), it is expected that changing the external calcium concentration will affect the repetitive discharge produced by DDT. When calcium concentration is raised to ten times normal in the presence of DDT, the negative after-potential is further augmented (Narahashi and Yamasaki, 1960b). However, the repetitive response is not suppressed by raising calcium concentration. Furthermore, in normal cockroach giant axons, lowering calcium concentration does not cause repetitive response. The negative after-potential is decreased by low-Ca, and increased by high-Ca. These results are not consistent with the hypothesis of Welsh and Gordon. It seems likely that the relationship between membrane calcium and DDT action, even though it does exist, is not as simple as assumed by them. It is required to re-investigate this problem in more detail by micro-electrode technique.

Unlike large axons such as the cockroach giant axons, small axons may give rise to prolonged repetitive discharge under the influence of DDT (Yamasaki and Ishii, 1952a). This study was made with peripheral nerves from the larva of a large beetle, *Allomyrina dichotomus*, by means of external electrodes. The diameters of the nerve fibers were not measured, but they must be very small in view of their slow conduction velocities (0·85–1·07 msec). After application of DDT, there appears a repetitive discharge in response to a single stimulus. The duration of repetitive discharge is prolonged with time, and finally trains of impulses appear spontaneously. Sometimes small oscillatory potential changes are observed. Since it is generally believed that repetitive responses are more easily elicited in smaller axons than in larger axons, the nerve preparation

used here seems to be suitable to analyse repetitive response in relation to DDT action. This preparation, though it is a multi-fiber nerve trunk, is available in a relatively long stretch (15–20 mm) without any ganglion intervening. It is hoped that analysis of repetitive response will be performed using this sort of nerve preparation.

3. *Negative after-potential*

During the course of our study on synaptic transmission and spontaneous activity in the DDT-poisoned cockroach nerve, it was noticed that the negative after-potential is markedly prolonged in duration (Yamasaki and Ishii, 1952b). A slight increase in negative after-potential had already been found in the crab nerve (Shanes, 1949b), whereas no such change was noted in squid and frog nerves (Shanes, 1949a, 1951b).

Later the negative after-potential under the influence of DDT was analysed by recording action potentials from single motor axons of the crural nerve and from the abdominal nerve cord by means of external electrodes (Yamasaki and Narahashi, 1957b). In the normal nerve, the spike potential is followed by a positive phase, and no supernormal phase occurs during the recovery after an impulse. On the contrary, there appears a marked supernormal phase following an impulse in the DDT-poisoned nerve, its time course being matched by a negative after-potential.

It was, however, not until analyses by means of micro-electrode technique became available that the mechanism underlying the negative after-potential was elucidated. The first preliminary observation of the negative after-potential by micro-electrodes was made in the same year (Yamasaki and Narahashi, 1957c). It was found later that the large negative after-potential in DDT is explicable in terms of changes in ionic conductances of the membrane (Narahashi and Yamasaki, 1960b, c).

After treatment with DDT, the spike phase of the action potential undergoes little change in spite of the increase in negative after-potential. As has been discussed in a preceding section (VI, A, 2), there is a brief period during which repetitive discharge is elicited by a single shock. At this stage of poisoning, the negative after-potential is in a slightly augmented state. After this period is over, the negative after-potential continues to increase and finally attains a maximum over many hours, but conduction is never impaired. If the large negative after-potential were due to an accumulation of a depolarizing substance such as potassium ions in the immediate vicinity of the membrane, there should be a build-up of the negative after-potentials on repetitive stimulation. Figure 22 shows records of the action potential and the negative after-potential produced by repetitive stimulation. It will be seen that there is little, if any,

build-up of the negative after-potentials, the result excluding the possibility of accumulation of depolarizing substance near the membrane. It was found that the delayed rectification observable in the normal membrane is greatly suppressed and further delayed by treatment with DDT.

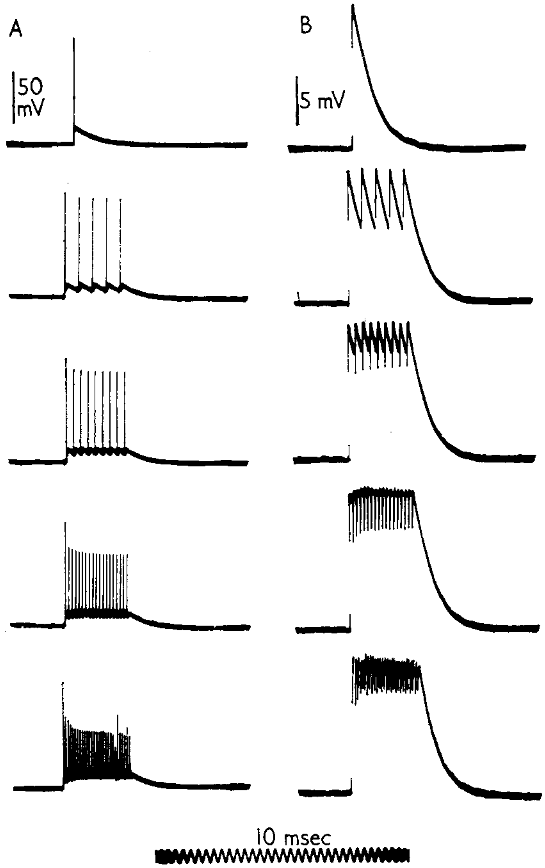

Fig. 22. Records of the action potentials induced by a single shock and by repetitive stimuli in the cockroach giant axon at various frequencies. 50–62 min after treatment with 10^{-4} M DDT. A, from top to bottom, single stimulus, 50, 100, 200 and 300 c/s. B, as in A, but with higher amplification (from Narahashi and Yamasaki, 1960b).

Since the rise in potassium conductance in normal nerve occurs with a delay and is sustained as long as the membrane is kept depolarized, the delayed rectification can be regarded as being indicative of the potassium conductance rise. It follows that the suppression and further delay of rectification is a reflexion of inhibition of the potassium conductance rise

by DDT. This should cause an increase in negative after-potential as has been observed.

It was also found that the large negative after-potential in DDT is further augmented by removing potassium from the external fluid, forming a plateau phase (Fig. 23). Increasing potassium concentration causes a decrease in negative after-potential. The axon which gives rise to action potentials having a plateau in K-free DDT medium shows the following

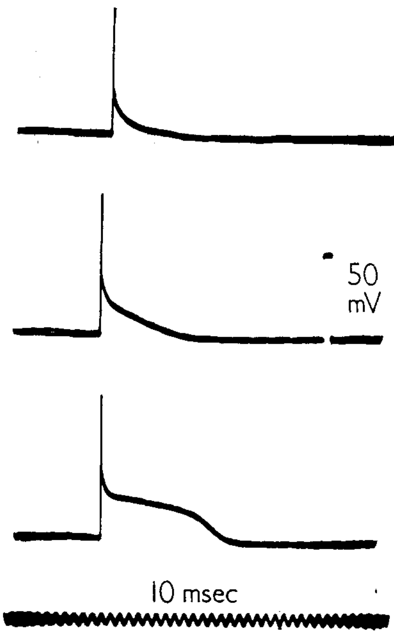

FIG. 23. Effect of changing the external potassium concentration on the action potential of the DDT-poisoned cockroach giant axon. 85–112 min after treatment with 10^{-4} M DDT. Top record, 10 mM K; middle record, 3·1 mM K; bottom record, 0 mM K (from Narahashi and Yamasaki, 1960b).

peculiar electrical behavior which is not seen in the normal axon: (1) Both critical depolarization and threshold current are greatly reduced. Since the resting potential undergoes little change, firing occurs at a higher membrane potential. (2) Break excitation is elicited by a very weak anodal current (Fig. 24). In the normal axon, no break excitation occurs even with a fairly strong anodal current. (3) Graded response is easily elicited upon small depolarization and upon breaking small hyperpolarization. The time course of the graded response is very much prolonged. (4) Spontaneous excitation may occur. In this case each action potential is pre-

ceded by a slowly rising phase. On some occasions graded potentials similar to those induced by stimulation are observed to occur spontaneously. (5) The plateau of the action potential can be abolished by applying a strong anodal pulse (Fig. 25). When a weak pulse is applied

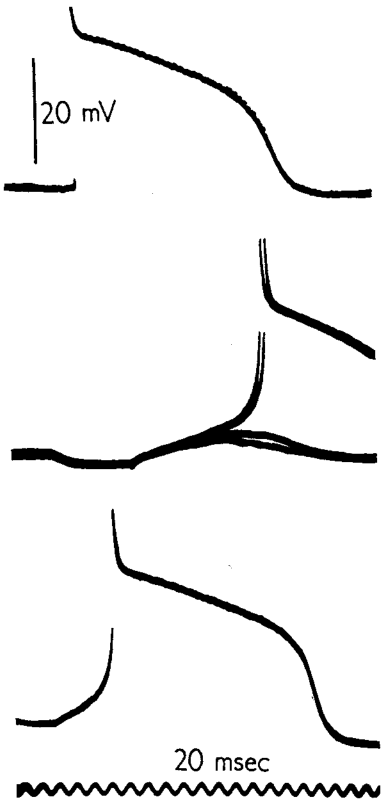

FIG. 24. Action potentials induced by a single shock applied to the end of the nerve preparation (top record), by pulses of anodal current applied to the point of recording (middle superimposed record), and by a pulse of cathodal current applied to the point of recording (bottom record) in potassium-free DDT solution. Cockroach giant axon. 122–138 min after treatment with DDT. The spike potentials are too large to be recorded (from Narahashi and Yamasaki, 1960c).

during the course of the plateau, the membrane potential returns to the level which is expected from the normal declining phase. With a stronger anodal pulse, however, the membrane potential does not return to the level expected, but stays at the resting level. With further increase in current strength, break response may occur. (6) The relative refractory period for the spike height ends at the moment when the plateau phase is

terminated, whereas the relative refractory period for the action potential duration lasts for a longer time after the completion of repolarization. The refractoriness for the action potential duration is abolished by an anodal pulse together with the plateau phase.

It is interesting to compare these electrical properties in the DDT-poisoned cockroach axons with those in cardiac muscle fibers and in squid giant axons treated with tetraethylammonium (TEA), in view of

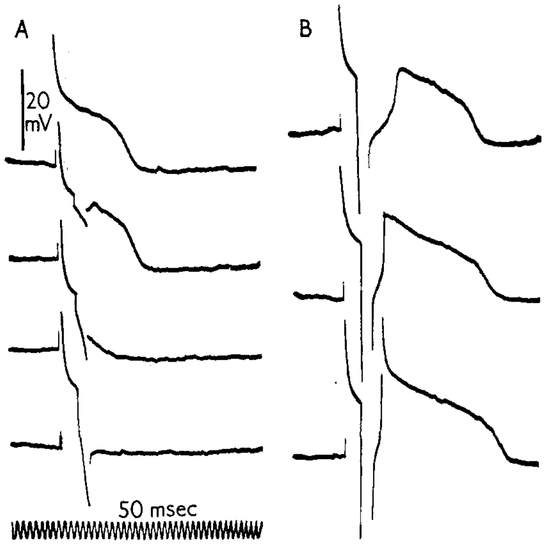

FIG. 25. Abolition of the plateau phase by anodal current and anode break excitation in potassium-free DDT solution. Cockroach giant axon 165 min after treatment with DDT. The anodal current is delivered at a fixed instant during the plateau and increased in intensity from the second record in column A to the bottom record in column B. The top record in A is an action potential without superimposed anodal pulse. Spike potentials are too large to be recorded (from Narahashi and Yamasaki, 1960c).

the resemblance of the action potential configuration. The slow graded response is also observed with cardiac muscle fibers in normal and in low-Ca conditions (Hoffman and Suckling, 1956; Hoffman *et al.*, 1957; Kao and Hoffman, 1958). The slowly rising potential preceding the spike is usually seen in cardiac and skeletal muscle fibers when spontaneously active (Bülbring *et al.*, 1956; Hoffman and Suckling, 1956; Weidmann, 1951b; Trautwein and Kassebaum, 1961). Abolition of the action potential by anodal pulse is observed with the nodes of Ranvier (Tasaki, 1956), the TEA-treated squid axons (Tasaki and Hagiwara, 1957), and with cardiac muscle fibers (Weidmann, 1957). The abolition of refractoriness during the plateau phase is in agreement with that observed with the

TEA-treated squid axons (Tasaki and Hagiwara, 1957). It can safely be said from these comparisons that the properties of the cockroach giant axon are changed to resemble those of cardiac muscle fibers as in the case of the TEA-treated squid giant axons.

The plateau phase seen in K-free DDT medium can be explained in terms of changes in potassium conductance rise and in sodium inactivation. If we assume the role of chloride ions in membrane potential to be negligible in the cockroach giant axons, equation (3) in Section III, B, 2 can be simplified as follows:

$$E_m = \frac{RT}{F}\log_e\frac{P_K[K]_o+P_{Na}[Na]_o}{P_K[K]_i+P_{Na}[Na]_i}. \tag{16}$$

The values for $[K]_i$ and $[Na]_i$ in the cockroach nerve are estimated as 140 and 84 mM respectively (Tobias, 1948a), and $[K]_o$ and $[Na]_o$ are 3·1 and 214 mM respectively. Thus in a condition satisfying $P_K = P_{Na}$, E_m becomes nearly zero. The height of the plateau is of the order of 30 mV or more. This level of potential would be maintained if P_K is somewhat higher than P_{Na}. This condition would be brought about after a spike potential either (a) by a fall of P_{Na} keeping P_K unchanged, or (b) by a fall of P_{Na} to a lesser extent accompanied by a small rise in P_K. After this, the membrane potential would decline slowly forming a plateau, if the ratio P_K/P_{Na} were raised very slowly. The membrane resistance is shown to be of appreciable value immediately after the spike and to increase slowly during the plateau phase, the observation being rather in favor of the view (a) above.

The termination of the plateau can also be explained. There are three possible explanations: (a) Fall of P_{Na} to the resting level; (b) rise in $[K]_o$ near the membrane; (c) rise in P_K. The presence of a positive phase which follows the plateau rules out the possibility (a) above. The rise in $[K]_o$ is shown to shorten the plateau (Fig. 23), but it is difficult to explain the sudden repolarization, because the increase in $[K]_o$ is thought to occur progressively. The explanation (c) above seems satisfactory, because the delay of potassium conductance rise in DDT is shown as described before, and because this explains the positive phase which follows the plateau.

It is of great interest that computations based on the Hodgkin-Huxley equation (Hodgkin, 1958; Hodgkin and Huxley, 1952d) have resulted in an action potential having a plateau in its falling phase (FitzHugh, 1960; George and Johnson, 1961; Noble, 1962). By modifying the time constants of sodium inactivation and potassium activation, it is possible to mimic the cardiac action potential and the TEA-induced action potential. Computations are successful not only in reproducing the plateau phase

but also in reproducing the positive phase following the plateau, abolition by anodal pulse, long refractory period, anode break excitation, and the pace-maker potential preceding the spike. These results of computation support the explanation for the DDT-induced plateau described here.

B. PYRETHRINS AND ALLETHRIN

Pyrethrins cause repetitive discharge and conduction block (Welsh and Gordon, 1947; Yamasaki and Ishii, 1952a). Micro-electrode analyses

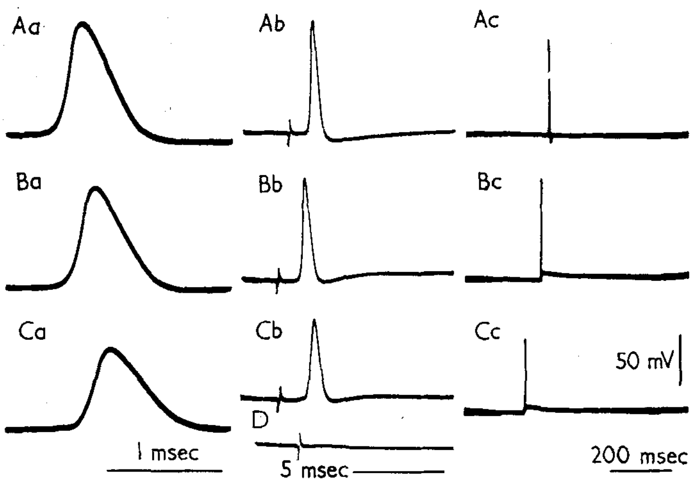

FIG. 26. Action potentials of the cockroach giant axon recorded before (A, a–c), 2 min 30 sec after (B, a–c), 4 min after (C, a–c) and 10 min 30 sec after (D) treatment with 10^{-6} g/ml allethrin (from Narahashi, 1962a).

were made using allethrin which is a synthetic derivative of pyrethrins (Narahashi, 1961 b, 1962 a, b).

After treatment with allethrin, the negative after-potential is increased. The positive phase following the spike is still observed as a dip between the spike potential and the augmented negative after-potential. When higher concentrations are used, conduction is eventually blocked (Fig. 26). In some cases there appears a repetitive after-discharge following an action potential. It was found that appearance of repetitive discharge depends on temperature. There is a critical temperature below which no repetitive discharge is elicited (Fig. 27). It is interesting that the action potentials are reduced in height progressively during repetitive discharge, and are followed by oscillations of small amplitudes. When repetitive discharge is stopped by lowering the temperature, an action potential is

directly followed by a damped oscillation. Unlike the DDT-poisoned axon, the condition of repetitive excitation lasts for a relatively long time unless the concentration of allethrin is high enough to block conduction.

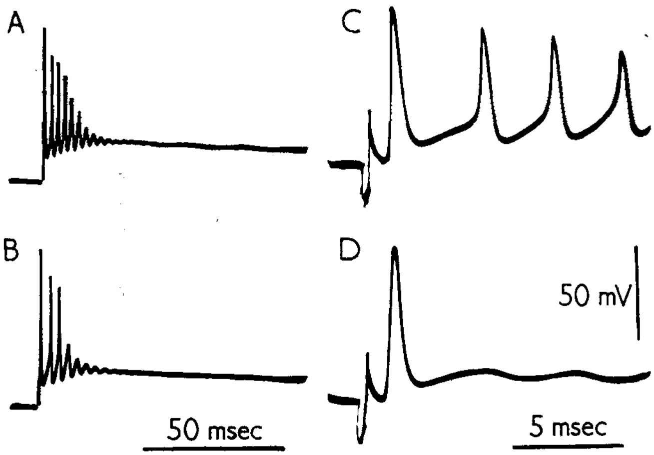

FIG. 27. Action potentials of the cockroach giant axon evoked by a single shock. 3×10^{-7} g/ml allethrin. Temperature: A, 33°C; B, 28°C; C, 26·5°C; D, 26°C (from Narahashi, 1962a).

Under the influence of allethrin, there is a build-up of the negative after-potentials upon repetitive stimuli (Fig. 28). This means that the large negative after-potential is produced by an accumulation of some depolarized substance inside or outside the nerve membrane. The falling phase

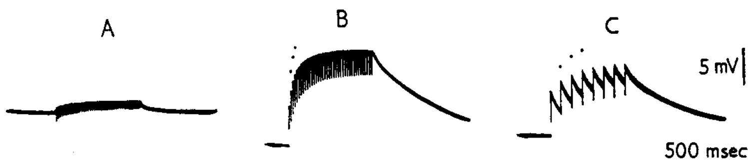

FIG. 28. Trains of impulses evoked by repetitive stimuli in the cockroach giant axon. Only the positive phase and the negative after-potential are seen; the spike potential is too large to be recorded. A, in normal Ringer's solution, 50 c/s; B, 10 min after treatment with 3×10^{-7} g/ml allethrin, 50 c/s; C, 11 min after treatment, 10 c/s. The dots drawn on the records B and C are the calculated heights of the second, third and fourth negative after-potential when the height of the first negative after-potential is added to each potential remaining at the moment when the next spike arises. In normal Ringer's solution, the calculated values are in good agreement with the observed ones (A) (from Narahashi, 1962b).

of the negative after-potential is very slow, the time constant being more than 100 msec. The membrane resistance undergoes little change by allethrin and the time constant of the normal membrane is estimated as 4·2 msec (Yamasaki and Narahashi, 1959b). It follows that the mechanism

producing the large negative after-potential is eliminated very slowly during the falling phase, and that the falling phase is hardly governed by the membrane time constant. This view is supported by the observation that the rate of fall of the large negative after-potential is little affected by high-K media which are known to reduce the membrane resistance. This result is in striking contrast with that of normal axons, in which the time constant of the falling phase of the negative after-potential is only about twice as long as the membrane time constant, so that increasing potassium concentration does accelerate the falling phase of the negative after-potential (Narahashi and Yamasaki, 1960a).

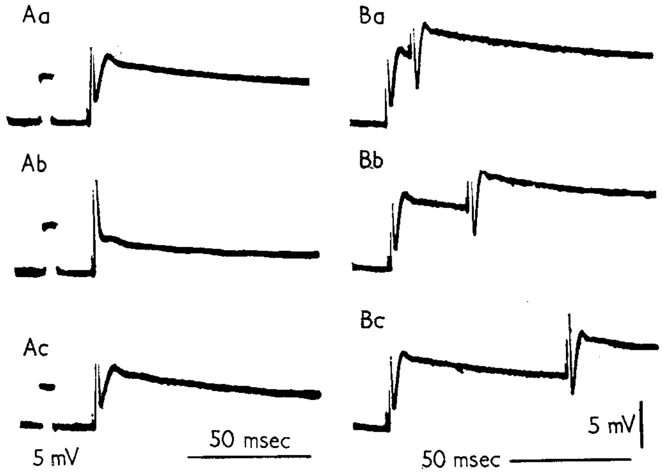

FIG. 29. Changes in positive phase and negative after-potential by K-rich solution (A) and by the negative after-potential of the conditioning impulse (B) in the allethrin-treated cockroach giant axon. The spike potential is too large to be recorded. Aa, in 10^{-7} g/ml allethrin; Ab, after treatment with 30 mM K; Ac, after bringing back to 10^{-7} g/ml allethrin. B, a–c, the conditioning and the test impulses were evoked at varying intervals (from Narahashi, 1962b).

One of the possible depolarizing substances responsible for the large negative after-potential in the allethrin-poisoned axons is potassium. However, this possibility is excluded by the observation illustrated in Fig. 29. When the external potassium concentration is raised, the membrane is depolarized and the configuration of the post-spike phase is changed as is shown in record Ab. If the large negative after-potential were due to the accumulation of potassium near the membrane, the post-spike phase of the second impulse elicited during the course of a negative after-potential of the first impulse would mimic that in K-rich media. However, as is shown in records B, a–c, this is not the case.

It is of interest that a prolonged repolarizing phase is also produced in the absence of sodium in the external medium (Fig. 30). When NaCl is replaced by choline chloride, no action potential is produced, yet a prolonged repolarizing phase follows a large and sustained depolarization

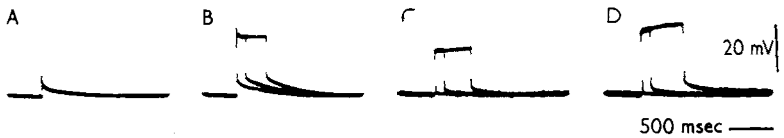

FIG. 30. Records of the negative after-potential following a spike potential and of the slowly repolarizing potential following a strong catelectrotonic potential in the cockroach giant axon. A, a single response showing a large and prolonged negative after-potential in the axon treated with 10^{-7} g/ml allethrin, the spike potential being too large to be recorded; B, superimposed records of the catelectrotonic potentials with varying durations produced by suprathreshold current, the spike potential being too large to be recorded; C, superimposed records of the catelectrotonic potentials with varying durations in the axon treated with 10^{-7} g/ml allethrin solution in which NaCl was replaced by choline chloride; D, the same as in C, but by stronger current (from Narahashi, 1962b).

produced by a cathodal current. It follows that the influx of sodium during excitation has no bearing on the large negative after-potential in allethrin. The negative after-potential is very sensitive to temperature change (Fig. 31). It is not only augmented in initial height but is also effectively shortened by a rise in temperature. This leads us to the view that

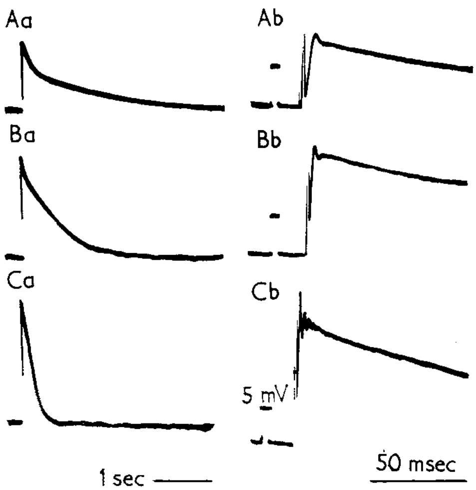

FIG. 31. Effect of temperature on the negative after-potential increased by 10^{-7} g/ml allethrin. Cockroach giant axon. The spike potential is too large to be recorded. Temperaure: Aa, 10°C; Ab, 15°C; Ba and Bb, 20°C; Ca, 27°C; Cb, 28°C (from Narahashi, 1962b).

some metabolic processes are involved in the large negative after-potential. It is concluded that the large negative after-potential under the influence of allethrin is produced by an accumulation of some depolarizing substance inside or outside the membrane, that this substance is neither potassium nor sodium, and that the substance is eliminated very slowly.

C. ROTENONE

It has been demonstrated that rotenone inhibits respiratory metabolism (Fukami, 1956, 1961; Fukami and Tomizawa, 1956; Lindahl and Öberg, 1961; Öberg, 1961). The inhibitory action of rotenone is due to interference with the electron-transport between reduced diphosphopyridine nucleotide and cytochrome b. Although the identification of the site of blocking in the respiratory chain was made with muscle, it is known that rotenone blocks conduction of nerves as well as in muscle. Meanwhile a number of rotenone derivatives became available. This situation provided an excellent opportunity to examine the relationship between inhibition of respiratory metabolism and block of nerve conduction (Fukami *et al.*, 1959). The inhibition of l-glutamic dehydrogenase by rotenone derivatives was estimated with the mitochondrial fraction of muscles of the beetle, *Allomyrina dichotomus*, while the ability to block nerve conduction was examined with the nerve cord of the cockroach. It was found that there is a good positive correlation between these actions; the derivatives which have a potent inhibitory action on respiratory metabolism block nerve conduction very effectively. Hence, it is concluded that the inhibition of respiratory metabolism is one of the main causes of conduction block.

ACKNOWLEDGEMENT

The author is greatly indebted to Dr. Julian M. Tobias for his valuable comment in preparing the manuscript.

REFERENCES

Araki, T. and Otani, T. (1955). *J. Neurophysiol.* **18**, 472–485. Response of single motoneurones to direct stimulation in toad's spinal cord.

Araki, T., Otani, T. and Furukawa, T. (1953). *Jap. J. Physiol.* **3**, 254–267. The electrical activities of single motoneurones in toad's spinal cord, recorded with intracellular electrodes.

Ashhurst, D. E. (1959). *Quart. J. micro. Sci.* **100**, 401–412. The connective tissue sheath of the locust nervous system: a histochemical study.

Asperen, K. van and Esch, I. van (1956). *Arch. néerl. Zool.* **11**, 342–360. The chemical composition of the haemolymph in *Periplaneta americana*.

Babers, F. H. (1938). *J. agric. Res.* **57**, 697–706. An analysis of the blood of the sixth-instar southern armyworm (*Prodenia eridania*).

Baccetti, B. (1957). *Exp. Cell Res.* **13**, 158–160. Observations by polarized light on the supporting stroma of some organs in insects.

Baerends, G. P. (1959). *Annu. Rev. Ent.* **4**, 207–234. Ethological studies of insect behavior.

Bernstein, J. (1912). "Elektrophysiologie", 215 pp., Fr. Vieweg u. Sohn, Braunschweig.

Bishop, G. H., Briggs, A. P. and Ronzoni, E. (1925). *J. biol. Chem.* **66**, 77–88. Body fluids of the honey bee larva. II. Chemical constituents of the blood, and their osmotic effects.

Boettiger, E. G. (1957). *In* "Recent Advances in Invertebrate Physiology", pp. 117–142 (eds. Scheer, B. T., Bullock, T. H., Kleinholz, L. H. and Martin, A. W.). University of Oregon, Eugene. The machinery of insect flight.

Boettiger, E. G. (1960). *Annu. Rev. Ent.* **5**, 1–16. Insect flight muscles and their basic physiology.

Boistel, J. (1960). "Caractéristiques Fonctionnelles des Fibres Nerveuses et des Récepteurs Tactiles et Olfactifs des Insectes", 147 pp. Librairie Arnette, Paris.

Boistel, J. and Coraboeuf, E. (1954). *C. R. Acad. Sci., Paris* **238**, 2116–2118. Potentiel de membrane et potentiels d'action de nerf d'insecte recueillis à l'aide de micro-électrodes intracellulaires.

Boné, G.-J. (1944). *Ann. Soc. zool. Belg.* **75**, 123–132. Le rapport sodium/potassium dans le liquide coelomique des insectes.

Boné, G.-J. (1946). *Nature, Lond.* **157**, 413. Sodium/potassium ratio in the coelomic fluid of insects.

Brebbia, D. R. and Ludwig, D. (1962). *Ann. Ent. Soc. Amer.* **55**, 131–135. Saline solutions for maintaining the isolated heart of the house fly, *Musca domestica* L.

Brecher, L. (1929). *Biochem. Z.* **211**, 40–64. Die anorganischen Bestandteile des Schmetterlingspuppenblutes (*Sphynx pinastri*, *Pieris brassicae*). Veränderungen im Gehalt an anorganischen Bestandteilen bei der Verpuppung (*Pieris brassicae*).

Brink, F. (1954). *Pharmacol. Rev.* **6**, 243–298. The role of calcium ions in neural processes.

Brock, L. G., Coombs, J. S. and Eccles, J. C. (1952). *J. Physiol.* **117**, 431–460. The recording of potentials from motoneurones with an intracellular electrode.

Bülbring, E., Holman, M. and Lüllmann, H. (1956). *J. Physiol.* **133**, 101–117. Effects of calcium deficiency on striated muscle of the frog.

Bullock, T. H. (1947). *Physiol. Rev.* **27**, 643–664. Problems in invertebrate electrophysiology.

Castillo, J. del, Hoyle, G. and Machne, X. (1953). *J. Physiol.* **121**, 539–547. Neuromuscular transmission in a locust.

Chadwick, L. E. (1953a). *In* "Insect Physiology", pp. 577–614 (ed. Roeder, K. D.). John Wiley & Sons, New York. The motion of the wings.

Chadwick, L. E. (1953b). *In* "Insect Physiology", pp. 615–636 (ed. Roeder, K. D.). John Wiley & Sons, New York. Aerodynamics and flight metabolism.

Chadwick, L. E. (1953c). *In* "Insect Physiology", pp. 637–655 (ed. Roeder, K. D.), John Wiley & Sons, New York. The flight muscles and their control.

Clark, E. W. and Craig, R. (1953). *Physiol. Zoöl.* **26**, 101–107. The calcium and magnesium content in the hemolymph of certain insects.

Cole, K. S. and Curtis, H. J. (1941). *J. gen. Physiol.* **24**, 551–563. Membrane potential of the squid giant axon during current flow.

Cole, K. S. and Hodgkin, A. L. (1939). *J. gen. Physiol.* **22**, 671–687. Membrane and protoplasm resistance in the squid giant axon.

Cook, P. M. (1951). *Quart. J. micr. Sci.* **92**, 297–305. Observations on giant fibres of the nervous system of *Locusta migratoria.*

Coraboeuf, E. and Boistel, J. (1955). *Colloq. int. Cent. nat. Rech. sci.* **67**, 57–72 (Microphysiologie Comparée des Éléments Excitables). Quelques aspects de la microphysiologie nerveuse chez les insectes.

Coraboeuf, E. and Weidmann, S. (1954). *Helv. physiol. acta* **12**, 32–41. Temperature effects on the electrical activity of Purkinje fibres.

Curtis, H. J. and Cole, K. S. (1940). *J. cell. comp. Physiol.* **15**, 147–157. Membrane action potentials from the squid giant axon.

Curtis, H. J. and Cole, K. S. (1942). *J. cell. comp. Physiol.* **19**, 135–144. Membrane resting and action potentials from the squid giant axon.

Dalton, J. C. (1958). *J. gen. Physiol.* **41**, 529–542. Effects of external ions on membrane potentials of a lobster giant axon.

Dalton, J. C. (1959). *J. gen. Physiol.* **42**, 971–982. Effects of external ions on membrane potentials of a crayfish giant axon.

Dethier, V. G. (1953a). *In* "Insect Physiology", pp. 488–522 (ed. Roeder, K. D.). John Wiley & Sons, New York. Vision.

Dethier, V. G. (1953b). *In* "Insect Physiology", pp. 523–543 (ed. Roeder, K. D.). John Wiley & Sons, New York. Mechanoreception.

Dethier, V. G. (1953c). *In* "Insect Physiology", pp. 544–576 (ed. Roeder, K. D.). John Wiley & Sons, New York. Chemoreception.

Draper, M. H. and Weidmann, S. (1951). *J. Physiol.* **115**, 74–94. Cardiac resting and action potentials recorded with an intracellular electrode.

Dresden, D. (1949). "Physiological Investigations into the Action of DDT". Drukkerij en Uitgeverij G. W. van der Wiel, Arnhem.

Drilhon, A. (1934). *C. R. Soc. Biol., Paris* **115**, 1194–1195. Sur le milieu interieur des Lépidoptères.

Duchâteau, G., Florkin, M. and Leclercq, J. (1953). *Arch. int. Physiol.* **61**, 518–549. Concentrations des bases fixes et types de composition de la base totale de l'hémolymphe des insectes.

Eccles, R. M. (1955). *J. Physiol.* **130**, 572–584. Intracellular potentials recorded from a mammalian sympathetic ganglion.

Edwards, G. A. (1957). *Anat. Rec.* **128**, 543. The fine structure of insect peripheral nerves and neuromuscular junctions.

Edwards, G. A. (1959). *J. biophys. biochem. Cytol.* **5**, 241–244. The fine structure of a multiterminal innervation of an insect muscle.

Edwards, G. A. (1960). *Annu. Rev. Ent.* **5**, 17–34. Insect micromorphology.

Edwards, G. A. and Challice, C. E. (1960). *Ann. ent. Soc. Amer.* **53**, 369–383. The ultrastructure of the heart of the cockroach, *Blattella germanica.*

Edwards, G. A., Ruska, H. and de Harven, É. (1958a). *J. biophys. biochem. Cytol.* **4**, 107–114. Electron microscopy of peripheral nerves and neuromuscular junctions in the wasp leg.

Edwards, G. A., Ruska, H. and de Harven, É. (1958b). *J. biophys. biochem. Cytol.* **4**, 251–256. Neuromuscular junctions in flight and tymbal muscles of the cicada.

Fatt, P. and Ginsborg, B. L. (1958). *J. Physiol.* **142**, 516–543. The ionic requirements for the production of action potentials in crustacean muscle fibres.

Fatt, P. and Katz, B. (1953). *J. Physiol.* **120**, 171–204. The electrical properties of crustacean muscle fibres.

Feng, T. P., Hsu, C. H. and Liu, Y. M. (1950). *Chin. J. Physiol.* **17**, 247–258. The Mechanism of the recovery of nerve asphyxiated in nitrogen when washed with O_2-free Ringer.

Fielden, A. (1960). *J. exp. Biol.* **37**, 832–844. Transmission through the last abdominal ganglion of the dragonfly nymph, *Anax imperator.*

FitzHugh, R. (1960). *J. gen. Physiol.* **43**, 867–896. Thresholds and plateaus in the Hodgkin-Huxley nerve equations.

Frank, G. B. (1957). *J. Neurophysiol.* **20**, 602–614. Negative after-potential of frog's skeletal muscle.

Frank, K. and Fuortes, M. G. F. (1956). *J. Physiol.* **134**, 451–470. Stimulation of spinal motoneurones with intracellular electrodes.

Frankenhaeuser, B. and Hodgkin, A. L. (1956). *J. Physiol.* **131**, 341–376. The after-effects of impulses in the giant nerve fibres of *Loligo.*

Frankenhaeuser, B. and Hodgkin, A. L. (1957). *J. Physiol.* **137**, 218–244. The action of calcium on the electrical properties of squid axons.

Frings, H. and Frings, M. (1958). *Annu. Rev. Ent.* **3**, 87–106. Uses of sounds by insects.

Fukami, J. (1956). *Botyu-Kagaku* **21**, 122–128. Effects of some insecticides on the respiration of insect organs, with special reference to the effects of rotenone.

Fukami, J. (1961). *Bull. Nat. Inst. Agr. Sci.* C, **13**, 33–45. Effect of rotenone on respiratory enzyme system of insect muscle.

Fukami, J. and Tomizawa, C. (1956). *Botyu-Kagaku* **21**, 129–133. Effects of rotenone on the 1-glutamic oxidase system in the insect.

Fukami, J., Nakatsugawa, T. and Narahashi, T. (1959). *Jap. J. appl. Ent. Zool.* **3**, 259–265. The relation between chemical structure and toxicity in rotenone derivatives.

Furukawa, T. (1953). *Jap. J. Physiol.* **3**, 268–276. Resting and action potential of single muscle fibres.

George, E. P. and Johnson, E. A. (1961). *Aust. J. exp. Biol. med. Sci.* **39**, 275–294. Solutions of the Hodgkin-Huxley equations for squid axon treated with tetraethylammonium and in potassium-rich media.

Glynn, I. M. (1956). *J. Physiol.* **134**, 278–310. Sodium and potassium movements in human red cells.

Gordon, H. T. and Welsh, J. H. (1948). *J. cell. comp. Physiol.* **31**, 395–419. The role of ions in axon surface reactions to toxic organic compounds.

Graham, J. and Gerard, R. W. (1946). *J. cell. comp. Physiol.* **28**, 99–117. Membrane potentials and excitation of impaled single muscle fibers.

Greengard, P. and Straub, R. W. (1958). *J. Physiol.* **144**, 442–462. After-potentials in mammalian non-myelinated nerve fibres.

Greengard, P. and Straub, R. W. (1959). *J. Physiol.* **145**, 562–569. Restoration by barium of action potentials in sodium-deprived mammalian B and C fibres.

Haapanen, L. and Ottoson, D. (1954). *Acta physiol. scand.* **32**, 271–280. A frequency compensated input unit for recording with microelectrodes.

Hagiwara, S. (1953). *Jap. J. Physiol.* **3**, 284–296. Neuro-muscular transmission in insects.

Hagiwara, S. and Watanabe, A. (1954). *Jap. J. Physiol.* **4**, 65–78. Action potential of insect muscle examined with intra-cellular electrode.

Hagiwara, S. and Watanabe, A. (1956). *J. cell. comp. Physiol.* **47**, 415–428. Discharges in motoneurons in cicada.

Harris, E. J. and Maizels, M. (1951). *J. Physiol.* **113**, 506–524. The permeability of human erythrocytes to sodium.

Hashimura, S. and Wright, E. B. (1958). *J. Neurophysiol.* **21**, 24–44. Effect of ionic environment on excitability and electrical properties of frog single nerve fiber.

Hess, A. (1958a). *J. Morph.* **103**, 479–501. Experimental anatomical studies of pathways in the severed central nerve cord of the cockroach.

Hess, A. (1958b). *J. biophys. biochem. Cytol.* **4**, 731–742. The fine structure of nerve cells and fibers, neuroglia, and sheaths of the ganglion chain in the cockroach. (*Periplaneta americana*).

Hess, A. (1958c). *Quart. J. micr. Sci.* **99**, 333–340. The fine structure and morphological organization of the peripheral nerve-fibres and trunks of the cockroach (*Periplaneta americana*).

Hill, A. V. (1928). *Proc. roy. Soc.* B **104**, 39–96. The diffusion of oxygen and lactic acid through tissues.

Hodgkin, A. L. (1947a). *J. Physiol.* **106**, 305–318. The membrane resistance of a non-medullated nerve fibre.

Hodgkin, A. L. (1947b). *J. Physiol.* **106**, 319–340. The effect of potassium on the surface membrane of an isolated axon.

Hodgkin, A. L. (1951). *Biol. Rev.* **26**, 339–409. The ionic basis of electrical activity in nerve and muscle.

Hodgkin, A. L. (1958). *Proc. roy. Soc.* B **148**, 1–37. Ionic movements and electrical activity in giant nerve fibres.

Hodgkin, A. L. and Huxley, A. F. (1939). *Nature, Lond.* **144**, 710–711. Action potentials recorded from inside a nerve fibre.

Hodgkin, A. L. and Huxley, A. F. (1945). *J. Physiol.* **104**, 176–195. Resting and action potentials in single nerve fibres.

Hodgkin, A. L. and Huxley, A. F. (1952a). *J. Physiol.* **116**, 449–472. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*.

Hodgkin, A. L. and Huxley, A. F. (1952b). *J. Physiol.* **116**, 473–496. The components of membrane conductance in the giant axon of *Loligo*.

Hodgkin, A. L. and Huxley, A. F. (1952c). *J. Physiol.* **116**, 497–506. The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*.

Hodgkin, A. L. and Huxley, A. F. (1952d). *J. Physiol.* **117**, 500–544. A quantitative description of membrane current and its application to conduction and excitation in nerve.

Hodgkin, A. L. and Katz, B. (1949a). *J. Physiol.* **108**, 37–77. The effect of sodium ions on the electrical activity of the giant axon of the squid.

Hodgkin, A. L. and Katz, B. (1949b). *J. Physiol.* **109**, 240–249. The effect of temperature on the electrical activity of the giant axon of the squid.

Hodgkin, A. L. and Keynes, R. D. (1955). *J. Physiol.* **128**, 28–60. Active transport of cations in giant axons from *Sepia* and *Loligo*.

Hodgkin, A. L. and Rushton, W. A. H. (1946). *Proc. roy. Soc.* B **133**, 444–479. The electrical constants of a crustacean nerve fibre.

Hodgkin, A. L., Huxley, A. F. and Katz, B. (1949). *Arch. Sci. physiol.* **3**, 129–150. Ionic currents underlying activity in the giant axon of the squid.

Hodgkin, A. L., Huxley, A. F. and Katz, B. (1952). *J. Physiol.* **116**, 424–448. Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*.

Hodgson, E. S. (1958). *Annu. Rev. Ent.* **3**, 19–36. Chemoreception in arthropods.

Hoffman, B. F. and Suckling, E. E. (1956). *Amer. J. Physiol.* **186**, 317–324. Effect of several cations on transmembrane potentials of cardiac muscle.

Hoffman, B. F., Kao, C. Y. and Suckling, E. E. (1957). *Amer. J. Physiol.* **190**, 473–482. Refractoriness in cardiac muscle.

Hoyle, G. (1952). *Nature, Lond.* **169**, 281–282. High blood potassium in insects in relation to nerve conduction.

Hoyle, G. (1953). *J. exp. Biol.* **30**, 121–135. Potassium ions and insect nerve muscle.

Hoyle, G. (1954a). *J. exp. Biol.* **31**, 260–270. Changes in the blood potassium concentration of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) during food deprivation, and the effect on neuromuscular activity.

Hoyle, G. (1954b). *Proc. roy. Soc.* B **143**, 281–292. The anatomy and innervation of locust skeletal muscle.

Hoyle, G. (1955). *J. Physiol.* **127**, 90–103. The effects of some common cations on neuromuscular transmission in insects.

Hoyle, G. (1957). "Comparative Physiology of the Nervous Control of Muscular Contraction", 147 pp. University Press, Cambridge.

Hughes, G. M. (1953). *Nature, Lond.* **171**, 87–88. "Giant" fibres in dragonfly nymphs.

Huxley, A. F. and Stämpfli, R. (1951a). *J. Physiol.* **112**, 476–495. Direct determination of membrane resting potential and action potential in single myelinated nerve fibres.

Huxley, A. F. and Stämpfli, R. (1951b). *J. Physiol.* **112**, 496–508. Effect of potassium and sodium ions on resting and action potentials of single myelinated nerve fibres.

Imms, A. D. (1957). "A General Textbook of Entomology", 9th ed., 886 pp. Methuen, London.

Ito, M. (1957). *Jap. J. Physiol.* **7**, 297–323. The electrical activity of spinal ganglion cells investigated with intracellular microelectrodes.

Kao, C. Y. and Hoffman, B. F. (1958). *Amer. J. Physiol.* **194**, 187–196. Graded and decremental response in heart muscle fibers.

Katz, B. (1948). *Proc. roy. Soc.* B **135**, 506–534. The electrical properties of the muscle fibre membrane.

Keynes, R. D. (1951). *J. Physiol.* **114**, 119–150. The ionic movements during nervous activity.

Keynes, R. D. (1954). *Proc. roy. Soc.* B **142**, 359–382. The ionic fluxes in frog muscle.

Keynes, R. D. and Lewis, P. R. (1951). *J. Physiol.* **114**, 151–182. The sodium and potassium content of cephalopod nerve fibres.

Keynes, R. D. and Swan, R. C. (1959). *J. Physiol.* **147**, 626–638. The permeability of frog muscle fibres to lithium ions.

Koechlin, B. A. (1955). *J. biophys. biochem. Cytol.* **1**, 511–529. On the chemical composition of the axoplasm of squid giant nerve fibers with particular reference to its ion pattern.

Koketsu, K. and Noda, K. (1960). *Nature, Lond.* **187**, 243–244. Restoration of membrane excitability of calcium-deficient muscles by anodal polarization.

Lalonde, D. I. V. and Brown, A. W. A. (1954). *Canad. J. Zool.* **32**, 74–81. The effect of insecticides on the action potentials of insect nerve.

Lehrman, D. S. (1956). *Annu. Rev. Physiol.* **18**, 527–542. Comparative physiology (behavior).

Li, C.-L. (1955). *J. Physiol.* **130**, 96–108. Action and resting potentials of cortical neurones.

Lindahl, P. E. and Öberg, K. E. (1961). *Exp. Cell Res.* **23**, 228–237. The effect of rotenone on respiration and its point of attack.

Ling, G. and Gerard, R. W. (1949a). *J. cell. comp. Physiol.* **34**, 383–396. The normal membrane potential of frog sartorius fibers.

Ling, G. and Gerard, R. W. (1949b). *J. cell. comp. Physiol.* **34**, 397–405. The influence of stretch on the membrane potential of the striated muscle fiber.

Ling, G. and Gerard, R. W. (1949c). *J. cell comp. Physiol.* **34**, 413–438. The membrane potential and metabolism of muscle fibers.

Ling, G. and Gerard, R. W. (1950). *Nature, Lond.* **165**, 113–114. External potassium and the membrane potential of single muscle fibres.

Ling, G. and Woodbury, J. W. (1949). *J. cell. comp. Physiol.* **34**, 407–412. Effect of temperature on the membrane potential of frog muscle fibers.

Lorente de Nó, R. (1947a). *Stud. Rockefeller Inst. Med. Res.* **131**, 1—496. A study of nerve physiology. Part 1.

Lorente de Nó, R. (1947b). *Stud. Rockefeller Inst. Med. Res.* **132**, 1–548. A study of nerve physiology. Part 2.

Ludwig, D., Tracey, S. K. M. and Burns, M.-L. (1957). *Ann. ent. Soc. Amer.* **50**, 244–246. Ratios of ions required to maintain the heart beat of the American cockroach, *Periplaneta americana Linnaeus.*

MacNichol, E. F. and Wagner, H. G. (1954). *Nav. med. res. Inst.* **12**, 97–118. A high impedance input circuit suitable for electrophysiological recording from micropipette electrodes.

Naka, K. and Kuwabara, M. (1959). *J. insect Physiol.* **3**, 41–49. Electrical response from the compound eye of *Lucilia.*

Narahashi, T. (1960). *In* "Electrical Activity of Single Cells", pp. 119–131 (ed. Katsuki, Y.). Igakushoin, Tokyo. Excitation and electrical properties of giant axon of cockroaches.

Narahashi, T. (1961a). *J. Physiol.* **156**, 389–414. Effect of barium ions on membrane potentials of cockroach giant axons.

Narahashi, T. (1961b). *Physiologist* **4**, 80. Nature of the negative after-potential increased by the insecticide allethrin in cockroach giant axons.

Narahashi, T. (1962a). *J. cell. comp. Physiol.* **59**, 61–65. Effect of the insecticide allethrin on membrane potentials of cockroach giant axons.

Narahashi, T. (1962b). *J. cell. comp. Physiol.* **59**, 67–76. Nature of the negative after-potential increased by the insecticide allethrin in cockroach giant axons.

Narahashi, T. and Yamasaki, T. (1960a). *J. Physiol.* **151**, 75–88. Mechanism of the after-potential production in the giant axons of the cockroach.

Narahashi, T. and Yamasaki, T. (1960b). *J. Physiol.* **152**, 122–140. Mechanism of increase in negative after-potential by dicophanum (DDT) in the giant axons of the cockroach.

Narahashi, T. and Yamasaki, T. (1960c). *J. cell. comp. Physiol.* **55**, 131–142. Behaviors of membrane potential in the cockroach giant axons poisoned by DDT.

Narahashi, T., Deguchi, T., Urakawa, N. and Ohkubo, Y. (1960). *Amer. J. Physiol.* **198**, 934–938. Stabilization and rectification of muscle fiber membrane by tetrodotoxin.

Nastuk, W. L. and Hodgkin, A. L. (1950). *J. cell. comp. Physiol.* **35**, 39–74. The electrical activity of single muscle fibers.

Nicholls, J. G. (1956). *J. Physiol.* **131**, 1–12. The electrical properties of denervated skeletal muscle.

Noble, D. (1962). *J. Physiol.* **160**, 317–352. A modification of the Hodgkin-Huxley equations applicable to Purkinje fibre action and pace-maker potentials.

Öberg, K. E. (1961). *Exp. Cell Res.* **24**, 163–164. The site of the action of rotenone in the respiratory chain.

O'Brien, R. D. (1957). *Ann. ent. Soc. Amer.* **50**, 223–229. Esterases in the semi-intact cockroach.

Overton, E. (1902). *Pflüg. Arch. ges. Physiol.* **92**, 346–386. Beiträge zur allgemeinen Muskel und Nervenphysiologie.

Posternak, J. and Arnold E. (1954). *J. Physiol., Paris* **46**, 502–505. Action de l'anélectrotonus et d'une solution hypersodique sur la conduction dans un nerf narcotisé.

Pringle, J. W. S. (1938). *J. exp. Biol.* **15**, 101–113. Proprioception in insects. I. A new type of mechanical receptor from the palps of the cockroach.

Pringle, J. W. S. (1957). "Insect Flight", 133 pp. University Press, Cambridge.

Prosser, C. L. (1946). *Physiol. Rev.* **26**, 337–382. The physiology of nervous systems of invertebrate animals.

Prosser, C. L. (1950a). *In* "Comparative Animal Physiology", pp. 576–629 (ed. Prosser, C. L.). W. B. Saunders Company, Philadelphia. Muscle and electric organs.

Prosser, C. L. (1950b). *In* "Comparative Animal Physiology", pp. 776–862 (ed. Prosser, C. L.). W. B. Saunders Company, Philadelphia. Nervous system.

Prosser, C. L. (1954). *Annu. Rev. Physiol.* **16**, 103–124. Comparative physiology of nervous systems and sense organs.

Pumphrey, R. J. and Rawdon-Smith, A. F. (1937). *Proc. roy. Soc.* B 122, 106–118. Synaptic transmission of nervous impulses through the last abdominal ganglion of the cockroach.

Ramsey, J. A. (1953). *J. exp. Biol.* **30**, 358–369. Active transport of potassium by the Malpighian tubules of insects.

Richards, A. G. (1944). *J. N.Y. ent. Soc.* **52**, 285–310. The structure of living insect nerves and nerve sheaths as deduced from the optical properties.

Roeder, K. D. (1948a). *J. exp. Zool.* **108**, 243–261. Organization of the ascending giant fiber system in the cockroach (*Periplaneta americana*).

Roeder, K. D. (1948b). *J. cell. comp. Physiol.* **31**, 327–338. The effect of potassium and calcium on the nervous system of the cockroach, *Periplaneta americana*.

Roeder, K. D. (1953a). *In* "Insect Physiology", pp. 423–462 (ed. Roeder, K. D.). John Wiley & Sons, New York. Electrical activity in nerves and ganglia.

Roeder, K. D. (1953b). *In* "Insect Physiology", pp. 463–487, (ed. Roeder, K. D.). John Wiley & Sons, New York. Reflex activity and ganglion function.

Roeder, K. D. (1958). *Annu. Rev. Ent.* **3**, 1–18. The nervous system.

Roeder, K. D. and Weiant, E. A. (1946). *Science*, **103**, 304–306. The site of action of DDT in the cockroach.

Roeder, K. D. and Weiant, E. A. (1948). *J. cell. comp. Physiol.* **32**, 175–186. The effect of DDT on sensory and motor structures in the cockroach leg.

Roeder, K. D., Kennedy, N. K. and Samson, E. A. (1947). *J. Neurophysiol.* **10**, 1–10. Synaptic conduction to giant fibers of the cockroach and the action of anticholinesterases.

Rudall, K. M. (1955). *Symp. Soc. exp. Biol.* **9**, 49–71. The distribution of collagen and chitin.

Scharrer, B. C. J. (1939). *J. comp. Neurol.* **70**, 77–88. The differentiation between neuroglia and connective tissue sheath in the cockroach (*Periplaneta americana*).

Schneider, K. C. (1902). "Lehrbuch der vergleichenden Histologie der Tiere", 988 pp. Fischer, Jena.

Schneirla, T. C. (1953a). *In* "Insect Physiology", pp. 656–684 (ed. Roeder, K. D.), John Wiley & Sons, New York. Basic problems in the nature of insect behavior.

Schneirla, T. C. (1953b). *In* "Insect Physiology", pp. 685–722 (ed. Roeder, K. D.), John Wiley & Sons, New York. Insect behavior in relation to its setting.

Schneirla, T. C. (1953c). *In* "Insect Physiology", pp. 723–747 (ed. Roeder, K. D.), John Wiley & Sons, New York. Modifiability in insect behavior.

Schoepfle, G. M. (1957). *Fed. Proc.* **16**, 114. Pentothal block of single nerve fibers and subsequent revival by means of anodal polarization.

Shanes, A. M. (1949a). *J. gen. Physiol.* **33**, 57–73. Electrical phenomena in nerve. I. Squid giant axon.

Shanes, A. M. (1949b). *J. gen. Physiol.* **33**, 75–102. Electrical phenomena in nerve. II. Crab nerve.

Shanes, A. M. (1951a). *J. gen. Physiol.* **34**, 795–807. Potassium movement in relation to nerve activity.

Shanes, A. M. (1951b). *J. cell. comp. Physiol.* **38**, 17–40. Electrical phenomena in nerve. III. Frog sciatic nerve.

Shanes, A. M. (1958a). *Pharmacol. Rev.* **10**, 59–164. Electrochemical aspects of physiological and pharmacological action in excitable cells. Part I. The resting cell and its alteration by extrinsic factors.

Shanes, A. M. (1958b). *Pharmacol. Rev.* **10**, 165–273. Electrochemical aspects of physiological and pharmacological action in excitable cells. Part II. The action potential and excitation.

Shanes, A. M. Freygang, W. H., Grundfest, H. and Amatniek, E. (1959). *J. gen. Physiol.* **42**, 793–802. Anesthetic and calcium action in the voltage clamped squid giant axon.

Shohl, A. T. (1939). "Mineral Metabolism", 384 pp. Reinhold Publishing Corp., New York.

Smith, D. S. and Treherne, J. E. (1963). *In* "Advances in Insect Physiology", (eds. Beament, J. W. L., Treherne, J. E. and Wigglesworth, V. B.), Academic Press, London. Functional aspects of the organization of the insect nervous system.

Smith, D. S. and Wigglesworth, V. B. (1959). *Nature, Lond.* **183**, 127–128. Collagen in the perilemma of insect nerve.

Solms, S. J., Nastuk, W. L. and Alexander, J. T. (1953). *Rev. sci. Instrum.* **24**, 960–967. Development of a high fidelity preamplifier for use in the recording of bioelectric potentials with intracellular electrodes.

Stämpfli, R. (1959). *Ann. N.Y. Acad. Sci.* **81**, 265–284. Is the resting potential of Ranvier nodes a potassium potential?

Steinbach, H. B. (1952). *Proc. nat. Acad. Sci., Wash.* **38**, 451–455. On the sodium and potassium balance of isolated frog muscle.

Steinbach, H. B. and Spiegelman, S. (1943). *J. cell. comp. Physiol.* **22**, 187–196. The sodium and potassium balance in squid nerve axoplasm.

Takeuchi, T. and Tasaki, I. (1942). *Pflüg. Arch. ges. Physiol.* **246**, 32–43. Übertragung des Nervenimpulses in der polarisierten Nervenfaser.

Tamasige, M. (1951). *Annot. Zool. Japon.* **24**, 141–149. Effect of potassium ions upon the electrical resistance of an isolated muscle fibre.

Tasaki, I. (1956). *J. gen. Physiol.* **39**, 377–396. Initiation and abolition of the action potential of a single node of Ranvier.

Tasaki, I. and Hagiwara, S. (1957). *J. gen. Physiol.* **40**, 859–885. Demonstration of two stable potential states in the squid giant axon under tetraethylammonium chloride.

Tasaki, I., Polley, E. H. and Orrego, F. (1954). *J. Neurophysiol.* **17**, 454–474. Action potentials from individual elements in cat geniculate and striate cortex.

Ten Cate, J. (1931). *Ergebn. Physiol.* **33**, 137–336. Physiologie der Gangliensysteme der Wirbellosen.

Tobias, J. M. (1948a). *J. cell. comp. Physiol.* **31**, 125—142. Potassium, sodium and water interchange in irritable tissues and haemolymph of an omnivorous insect, *Periplaneta americana*.

Tobias, J. M. (1948b). *J. cell. comp. Physiol.* **31**, 143–148. The high potassium and low sodium in the body fluid and tissues of a phytophagous insect, the silkworm *Bombyx mori* and the change before pupation.

Tobias, J. M. (1960). *J. gen. Physiol.* **43**, suppl. 57–71. Further studies on the nature of the excitable system in nerve. I. Voltage-induced axoplasm movement in squid axons. II. Penetration of surviving, excitable axons by proteases. III. Effects of proteases and of phospholipases on lobster giant axon resistance and capacity.

Tobias, J. M. and Bryant, S. H. (1955). *J. cell. comp. Physiol.* **46**, 163–182. An isolated giant axon preparation from the lobster nerve cord. Dissection, physical structure, trans-surface potentials and microinjection.

Tobias, J. M., Agin, D. P. and Pawlowski, R. (1962). *J. gen. Physiol.* **45**, 989–1001. Phospholipid-cholesterol membrane model. Control of resistance by ions or current flow.

Trautwein, W. and Kassebaum, D. G. (1961). *J. gen. Physiol.* **45**, 317–330. On the mechanism of spontaneous impulse generation in the pacemaker of the heart.

Trautwein, W., Gottstein, U. and Federschmidt, K. (1953). *Pflüg. Arch. ges. Physiol.* **258**, 243–260. Der Einfluss der Temperatur auf den Aktionsstrom des excidierten Purkinje-Fadens, gemessen mit einer intracellulären Elektrode.

Treherne, J. E. (1960). *J. exp. Biol.* **37**, 513–533. The nutrition of the central nervous system in the cockroach, *Periplaneta americana* L. The exchange and metabolism of sugars.

Treherne, J. E. (1961a). *J. exp. Biol.* **38**, 315–322. Sodium and potassium fluxes in the abdominal nerve cord of the cockroach, *Periplaneta americana* L.

Treherne, J. E. (1961b). *Nature, Lond.* **191**, 1223–1224. Exchanges of sodium ions in the central nervous system of an insect (*Periplaneta americana* L.).

Treherne, J. E. (1961c). *J. exp. Biol.* **38**, 629–636. The movements of sodium ions in the isolated abdominal nerve cord of the cockroach, *Periplaneta americana*.

Treherne, J. E. (1961d). *J. exp. Biol.* **38**, 729–736. The efflux of sodium ions from the last abdominal ganglion of the cockroach, *Periplaneta americana* L.

Treherne, J. E. (1961e). *J. exp. Biol.* **38**, 737–746. The kinetics of sodium transfer in the central nervous system of the cockroach, *Periplaneta americana* L.

Treherne, J. E. (1962). *Nature, Lond.* **193**, 750–752. Distribution of water and inorganic ions in the central nervous system of an insect (*Periplaneta americana* L.).

Twarog, B. M. and Roeder, K. D. (1956). *Biol. Bull., Woods Hole* **111**, 278–286. Properties of the connective tissue sheath of the cockroach abdominal nerve cord.

Twarog, B. M. and Roeder, K. D. (1957). *Ann. ent. Soc. Amer.* **50**, 231–237. Pharmacological observations on the desheathed last abdominal ganglion of the cockroach.

Ussing, H. H. (1954). *In* "Ion Transport Across Membranes", pp. 3–22 (eds. Clarke, H. T. and Nachmansohn, D.). Academic Press, New York. Ion transport across biological membranes.

Watanabe, A. and Grundfest, H. (1961). *J. gen. Physiol.* **45**, 267–308. Impulse propagation at the septal and commissural junctions of crayfish lateral giant axons.

Weidmann, S. (1951a). *J. Physiol.* **114**, 372–381. Electrical characteristics of *Sepia* axons.

Weidmann, S. (1951b). *J. Physiol.* **115**, 227–236. Effect of current flow on the membrane potential of cardiac muscle.

Weidmann, S. (1952). *J. Physiol.* **118**, 348–360. The electrical constants of Purkinje fibres.

Weidmann, S. (1955a). *J. Physiol.* **127**, 213–224. The effect of the cardiac membrane potential on the rapid availability of the sodium-carrying system.

Weidmann, S. (1955b). *J. Physiol.* **129**, 568–582. Effects of calcium ions and local anaesthetics on electrical properties of Purkinje fibres.

Weidmann, S. (1957). "Elektrophysiologie der Herzmuskelfaser", 2nd ed. Japanese translation by Matsuda, K. 106 pp. Kinhodo, Tokyo.

Welsh, J. H. and Gordon, H. T. (1947). *J. cell. comp. Physiol.* **30**, 147–171. The mode of action of certain insecticides on the arthropod nerve axon.

Welsh, J. H. and Schallek, W. (1946). *Physiol. Rev.* **26**, 447–478. Arthropod nervous systems: a review of their structure and function.

Werman, R. and Grundfest, H. (1961). *J. gen. Physiol.* **44**, 997–1027. Graded and all-or-none electrogenesis in arthropod muscle. II. The effects of alkali-earth and onium ions on lobster muscle fibers.

Werman, R., McCann, F. V. and Grundfest, H. (1961). *J. gen. Physiol.* **44**, 979–995. Graded and all-or-none electrogenesis in arthropod muscle. I. The effects of alkali-earth cations on the neuromuscular system of *Romalia microptera*.

Wiersma, C. A. G. (1952). *Annu. Rev. Physiol.* **14**, 159–176. Comparative physiology of invertebrate muscle.

Wiersma, C. A. G. (1953). *Physiol. Rev.* **33**, 326–355. Neural transmission in invertebrates.

Wigglesworth, V. B. (1956). *Quart. J. micr. Sci.* **97**, 89–98. The haemocytes and connective tissue formation in an insect, *Rhodnius prolixus* (Hemiptera).

Wigglesworth, V. B. (1959a). *Quart. J. micr. Sci.* **100**, 285–298. The histology of the nervous system of an insect, *Rhodnius prolixus* (Hemiptera). I. The peripheral nervous system.

Wigglesworth, V. B. (1959b). *Quart. J. micr. Sci.* **100**, 299–313. The histology of the nervous system of an insect, *Rhodnius prolixus* (Hemiptera). II. The central ganglia.

Wigglesworth, V. B. (1960). *J. exp. Biol.* **37**, 500–512. The nutrition of the central nervous system in the cockroach *Periplaneta americana* L. The role of the perineurium and glial cells in the mobilization of reserves.

Woronzow, D. S. (1924). *Pflüg. Arch. ges. Physiol.* **203**, 300–318. Über die Einwirkung des konstanten Stromes auf den mit Wasser, Zuckerlösung, Alkali- und Erdalkalichloridlösungen behandelten Nerven.

Wulff, V. J. (1956). *Physiol. Rev.* **36**, 145–163. Physiology of the compound eye.

Yamasaki, T. and Ishii, T. (1950a). *Ōyō-Kontyū* **5**, 155–168. Studies on the acting mechanism of insecticides (I) The actions of some sympatho- and parasympathomimetic drugs and insecticides on the heart of insect.

Yamasaki, T. and Ishii, T. (1950b). *Ōyō-Kontyū* **6**, 127–132. Studies on the acting mechanism of insecticides. (II) The action of DDT on the heart of insect, *Allomylina dichotomus* L.

Yamasaki, T. and Ishii, T. (1952a). *Ōyō-Kontyū* **7**, 157–164. Studies on the mechanism of action of insecticides. (IV) The effects of insecticides on the nerve conduction of insect.

Yamasaki, T. and Ishii, T. (1952b). *Ōyō-Kontyū* **8**, 111–118. Studies on the mechanism of action of insecticides. (V) The effects of DDT on the synaptic transmission in the cockroach.

Yamasaki, T. and Ishii, T. (1953). *Ōyō-Kontyū* **9**, 87–96. Studies on the mechanism of action of insecticides (VI). DDT symptoms in the cockroach, with special reference to the effect of temperature.

Yamasaki, T. and Ishii, T. (1954a). *Botyu-Kagaku* **19**, 1–14. Studies on the mechanism of action of insecticides (VII). Activity of neuron soma as a factor of development of DDT symptoms in the cockroach.

Yamasaki, T. and Ishii, T. (1954b). *Botyu-Kagaku* **19**, 39–46. Studies on the mechanism of action of insecticides (VIII). Effects of temperature on the nerve susceptibility to DDT in the cockroach.

Yamasaki, T. and Ishii, T. (1954c). *Jap. J. appl. Zool.* **19**, 16–28. Studies on the mechanism of action of insecticides (IX). Repetitive excitation of the insect neurone soma by direct current stimulation and effects of DDT.

Yamasaki, T. and Ishii, T. (1957a). *In* "Japanese Contributions to the Study of the Insecticide-Resistance Problem", pp. 130–139. Publ. by the Kyoto University for the W. H. O., Kyoto. Studies on the mechanism of action of insecticides (VI). DDT symptoms in the cockroach, with special reference to the effect of temperature.

Yamasaki, T. and Ishii, T. (1957b). *In* "Japanese Contributions to the Study of the Insecticide-Resistance Problem", pp. 140–154. Publ. by the Kyoto University for the W. H. O., Kyoto. Studies on the mechanism of action of insecticides (VII). Activity of neuron soma as a factor of development of DDT symptoms in the cockroach.

Yamasaki, T. and Ishii, T. (1957c). *In* "Japanese Contributions to the Study of the Insecticide-Resistance Problem", pp. 155–162. Publ. by the Kyoto University for the W.H.O., Kyoto. Studies on the mechanism of action of insecticides (VIII). Effects of temperature on the nerve susceptibility to DDT in the cockroach.

Yamasaki, T. and Ishii, T. (1957d). *In* "Japanese Contributions to the Study of the Insecticide-Resistance Problem", pp. 163–175. Publ. by the Kyoto University for the W. H. O., Kyoto. Studies on the mechanism of action of insecticides (IX). Repetitive excitation of the insect neurone soma by direct current stimulation and effects of DDT.

Yamasaki, T. and Narahashi, T. (1957a). *Botyu-Kagaku* **22**, 250–276. Effects of oxygen lack, metabolic inhibitors, and DDT on the resting potential of insect nerve. Studies on the mechanism of action of insecticides. XII.

Yamasaki, T. and Narahashi, T. (1957b). *Botyu-Kagaku* **22**, 296–304. Increase in the negative after-potential of insect nerve by DDT. Studies on the mechanism of action of insecticides. XIII.

Yamasaki, T. and Narahashi, T. (1957c). *Botyu-Kagaku* **22**, 305–313. Intracellular microelectrode recordings of resting and action potentials from the insect axon and the effects of DDT on the action potential. Studies on the mechanism of action of insecticides. XIV.

Yamasaki, T. and Narahashi, T. (1957d). *Botyu-Kagaku* **22**, 354–367. Effects of metabolic inhibitors, potassium ions and DDT on some electrical properties of insect nerve. Studies on the mechanism of action of insecticides. XV.

Yamasaki, T. and Narahashi, T. (1958a). *Botyu-Kagaku* **23**, 146–157. Resistance of house flies to insecticides and the susceptibility of nerve to insecticides. Studies on the mechanism of action of insecticides. XVII.

Yamasaki, T. and Narahashi, T. (1958b). *Nature, Lond.* **182**, 1805. Effects of potassium and sodium ions on the resting and action potentials of the giant axon of the cockroach.

Yamasaki, T. and Narahashi, T. (1959a). *J. insect Physiol.* **3**, 146–158. The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon.

Yamasaki, T. and Narahashi, T. (1959b). *J. insect Physiol.* **3**, 230–242. Electrical properties of the cockroach giant axon.

Yamasaki, T. and Narahashi, T. (1960). *J. insect Physiol.* **4**, 1–13. Synaptic transmission in the last abdominal ganglion of the cockroach.

Yeager, J. F. (1938). *J. agric. Res.* **56**, 267–276. Mechanographic method of recording insect cardiac activity, with reference to effect of nicotine on isolated heart preparations of *Periplaneta americana*.

Yeager, J. F. (1939). *J. agric. Res.* **59**, 121–137. Electrical stimulation of isolated heart preparations from *Periplaneta americana*.

The Chitin/Protein Complexes of Insect Cuticles

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I. INTRODUCTION

The insect cuticle is most simply viewed as consisting of a thin outer epicuticle, not containing chitin, overlying a more or less massive cuticle proper composed of chitin and protein. The epicuticle has attracted much attention because of its easily demonstrated physiological functions connected with water transfer and drug penetration. The remainder of the cuticle seems not to have very dramatic properties. A common and important feature is that the endocuticle is eroded during moulting indicating that it must be in a semi-stabilized state. However, the exocuticle frequently attains a very highly stable state due to the cross-linking of its protein structure, and cuticle substance in this condition would not be dissolved by the moulting fluids.

One tends to think in terms of hard and soft cuticles as if all hard

cuticles were about the same and that likewise soft cuticles all possessed similar properties. In contemplating the range of cuticle structures we may have two rather different views. In the first we may lay emphasis on the obviously great differences of sculpturing; some cuticles are rough surfaced, some smooth surfaced. The laminated texture may vary, with lamina thickness being in the range of 0·2–10 μ (Richards, 1951), yet one might suppose that at the molecular level cuticles were all much the same. Analysis of cuticles tells us of large quantities of the polysaccharide chitin and of protein, and one might suppose that these had a relatively standard structure in cuticles generally.

The opposite view would be that varying sculpturing and texture were not the major differences between cuticles, but rather that the really outstanding differences lay at the molecular or macromolecular level.

Analyses have told us that there is chitin and that there is protein in the cuticle. In themselves the data tell us nothing of any possible relationship between these two long-chain molecules. We have gradually learnt of several types of relationship between them and for the time being the term chitin/protein complex is used to refer to these relationships. The present article deals with molecular features of chitin/protein complexes with a view to revealing their variety. We are able to define types associated with particular kinds of cuticle and occasionally types are related to taxonomic groups.

In describing our main findings about chitin/protein complexes we are concerned with facts about chitin itself and facts about the protein which is associated with the chitin. Undoubtedly we have a pleasing variety of information on all these points. But there are some strange and conflicting matters which we will dwell upon, and if we can resolve these we may find satisfactory meaning in the variety of cuticle structures at the molecular level.

II. The Structure of Chitin

All early studies of chitin were made out of curiosity to answer the question "What sort of stuff is this?" Each time it was examined over the last hundred years or so, something new was found out—some immediate question about it was answered, such as its constitution, its density, its tensile strength, its solubility, its X-ray diffraction pattern, its infra-red absorption. Some seem to think that these studies have lead to an effectively complete knowledge of the structure of purified chitin. However, it is very possible that the things we do not know about chitin are just those which are most significant in relation to an understanding

of chitin/protein complexes. In searching for these missing facts, we will consider where chemical analysis, X-ray analysis and infra-red absorption really can be said to agree on one picture of the structure.

A. CHEMICAL ANALYSIS

The excellent review by Richards (1951) reflects the then current view that chitin was poly-*N*-acetylglucosamine. Tracey (1955a) seems to accept the probability of such a polymer but notes that "a preparation that contains the theoretical amount of nitrogen and acetyl groups is a rarity owing to the difficulties of purification without degradation". Nevertheless, it has often been assumed that the situation is satisfactory enough and that chitin is essentially polyacetylglucosamine. In particular, X-ray crystallographers have used this constitution with apparent success in deriving their structures.

A new and rather independent approach to the chemistry of chitin has been made by Giles *et al.* (1958). They attempt to remove protein and other substances leaving the chitin in an unmodified condition, seeking principally to avoid any deacetylation. The milder methods they use to remove protein are either pepsin hydrolysis (8 days at 37°C Buffer pH 1·4) or a modification of a so-called Thor process which entails boiling for 8 h in 1% aqueous sodium carbonate containing 0·2% anionic detergent. There are other mild treatments as well, i.e. in cold 5% aqueous hydrochloric acid. The material was well washed in water, dried and conditioned in air. Subsequent analyses refer to chitin samples which had been dried *in vacuo* over phosphorus pentoxide at 110°C for 36 h. The normal moisture content (calculated by drying to constant weight at 105°C) is about 7·5–9%.

These authors (Giles *et al.*) present analyses of the elements C, H, N for each of seven samples of purified chitin from two kinds of lobster; the means of these analyses are compared with the theoretical values for poly-*N*-acetylglucosamine. There is a considerable difference and so all possible mixtures of poly-*N*-acetylglucosamine, polyglucosamine and water, were considered to find the best agreement with the measured analyses. In parts by weight this was found to be:

Poly-*N*-acetylglucosamine	82·5%
Polyglucosamine	12·5%
Water	5·0%

This would give a picture of chitin structure with, say, every sixth or seventh residue along the chain being glucosamine instead of *N*-acetylglucosamine like the other residues. In addition a considerable

amount of strongly bound or trapped water would be a part of the structure.

If, for a given type of chitin, numerous separate analyses of elements gave these mean values, then one would accept the mixture of Giles *et al.* as a possible interpretation. It so happens that this view does seem to fit in with other information, but one feels sceptical of so direct an interpretation of the analysis of elements. We shall come to these possible supporting points in dealing with infra-red absorption and some aspects of X-ray analysis. Nevertheless, we should have expected clues to be forthcoming from the ratio of acetylglucosamine to glucosamine in the enzymic hydrolysis of chitin.

We might, first of all, remark on one conclusion which Giles *et al.* make from their elementary analysis of chitin which had been immersed in aqueous hydrochloric acid at 60°C for 24 h; they used three concentrations of acid with the final pH values 2·5 and 2·0 or with the initial concentration 2N. In the latter, the nitrogen content fell to 3·7% and they concluded that there was considerable loss of the whole acetamido group. It does not seem that even in the acid hydrolyses of chitins we are destroying glucosamine to the extent that this figure indicates. Furthermore, the infra-red absorption results of Pearson *et al.*, 1960, and Marchessault *et al.*, 1960, do not indicate any such drastic change in the case of their isolated crab crystallites which had been produced by way of treatment in 2·5N HCl for 40 min under reflux.

But to return to the main question at issue. What is the extent of the occurrence of glucosamine instead of *N*-acetylglucosamine in chitin structures? Histochemical studies are full of suggestions that some residues in chitins are deacetylated. Richards (1952) describes an intense red colour in the P.A.S. test on purified chitin provided that the sample has not been dried in air. Of course, as Brunet (1952) points out, this could result from some deacetylation during the purification in hot 10% NaOH solution. Nevertheless, we note that the P.A.S. reaction is described as intense and that the reaction is masked as a result of drying, as if the glucosamine units were now inaccessible within the crystalline regions of the structure. By similar histochemical tests Runham (1961 a, b) considers that chitin in the very long radula of *Patella* gives a strongly positive P.A.S. reaction only in the most proximal region, that is, where the chitin is newly formed and perhaps less aggregated. In *Rhodnius*, Wigglesworth (1956) finds the P.A.S. reaction to be negative in the endocuticle over the greater part of the body, but it is strongly positive in the flexible membrane of the neck and in the conjuntivae of the limbs. These tests may be considered too unreliable to lead to any

definite conclusions. Nevertheless, they do suggest the possibility of glucosamine residues, and that on drying these may become situated in crystalline or otherwise inaccessible regions of the structure.

Ideally we ought to be able to detect deacetylation in the infra-red absorption spectrum, either by the presence of $-NH_2$ groups or by changes in carbonyl absorption at about 1650 cm^{-1}. Neither of these regions of the spectrum readily lends itself to such a study and we will see later that the 1650 cm^{-1} region is particularly complex.

There remains direct chemical analysis, and Waterhouse *et al.* (1961) have attempted this using chitinase. They were very careful to avoid deacetylation of the substrate; for the main experiments they used crab chitin decalcified with EDTA (ethylenediaminetetra-acetic acid). They incubate this chitin for 6 days with various chitinase preparations from the cockroach and sometimes about 10% of the substrate is digested in that time. On the whole, rather small quantities of glucosamine are recorded in the digests, by far the major product being *N*-acetyl-glucosamine. Nevertheless, their Table 7 indicates that glucosamine may be present in quantities up to about 10% of the acetylglucosamine, i.e. 43 : 466 according to the data in which enzymes from cast skins were used. Cast skins themselves are stated in Table 8 to contain obviously some *N*-acetylglucosamine and only possibly a trace of glucosamine, while the dialysed enzyme used is free of these sugars. While deacetylase activity is said to be effectively absent from the blood, digestive juice and cuticle brei of the cockroach, no tests are reported in the case of cast skin extracts. However, their Table 8 does not suggest its presence there.

While these studies are really establishing the presence of glucosamine in chitins, there is difficulty in assessing the relative quantity of this. Tracey (1955b) gives a very obvious example of our difficulty. He was studying the digestion of chitosan by an active chitinase extract from the puffball *Lycoperdon giganteum*. Although this chitosan preparation had the probable constitution of 48% glucosamine residues: 42% acetyl-glucosamine, "the amount of glucosamine present after prolonged enzymic hydrolysis of the chitosan was certainly less than 10% of the acetylglucosamine and was probably nil".

In view of these experiments, it remains possible that considerable quantities of glucosamine could be present in the chains of native chitins. To a first approximation the missing acetyl groups could be replaced by water molecules, i.e. about two water molecules per acetyl group, going some way to account for the bound water fraction postulated by Giles *et al.* (1958).

B. X-RAY ANALYSIS

Attempts at relatively complete determinations of structure have all been made on chitin which has been purified by boiling in caustic alkali solutions (5 or 10%) for periods up to 48 h. Most of these studies have been on the tendons or apodemes of lobsters; such tendons also need to be decalcified in cold dilute hydrochloric acid. The chitin of these apodemes is better oriented and more highly crystalline than that of most other cuticular structures; no modern detailed chemical analyses are available for these particularly crystalline chitins.

1. *The different types of chitin: α, β, γ*

There are three main crystal structures among chitins and a consideration of their general features points directly to matters of biological interest. So we will begin by surveying these in a broadly comparative way before dealing with any details of structure.

Two of the crystal structures are already well known, there being the α-chitin structure, perhaps universally present in arthropod cuticles and apodemes, and the less common β-chitin, which was recognized by Lotmar and Picken (1950) as a distinct structure and found by them in *Aphrodite* chaetae and in the pen of the squid, *Loligo*. Yet a third distinct form of chitin has been recognized, Rudall (1962a), and, in fact, all three chitins may occur together in the same animal, but in different tissues.

The X-ray diffraction diagrams of the three chitin structures are shown side by side in Fig. 1, and were given by purified fibrous chitin from different organs of the squid *Loligo*. A is from the large skeletal pen, B is from the relatively thin oesophageal cuticle lining the stomach, and C is from the thick cuticle lining the stomach. The diagrams A, B and C of Fig. 1 show main features in common. Along the fibre axis (vertical) there are three main layer lines of very nearly the same position in each diagram. Laterally there are two particularly strong row lines (a) and (c) which depict the main separation of the chitin chains in planes parallel to the sugar ring (a) or in planes perpendicular to the sugar ring (c). These are the very obvious features which are common. The features which are special to each diagram concern the series of related row lines 1; 1,2,3; 1,2,3,4. In A there is a single row line 1, with no good evidence for any related row line nearer the centre. But in B there is a series of related row lines 1,2,3, while in C there are easily definable row lines 1,2,3,4. In A, B and C the equivalent row line (c) represents the first order of the structure in A, the second order

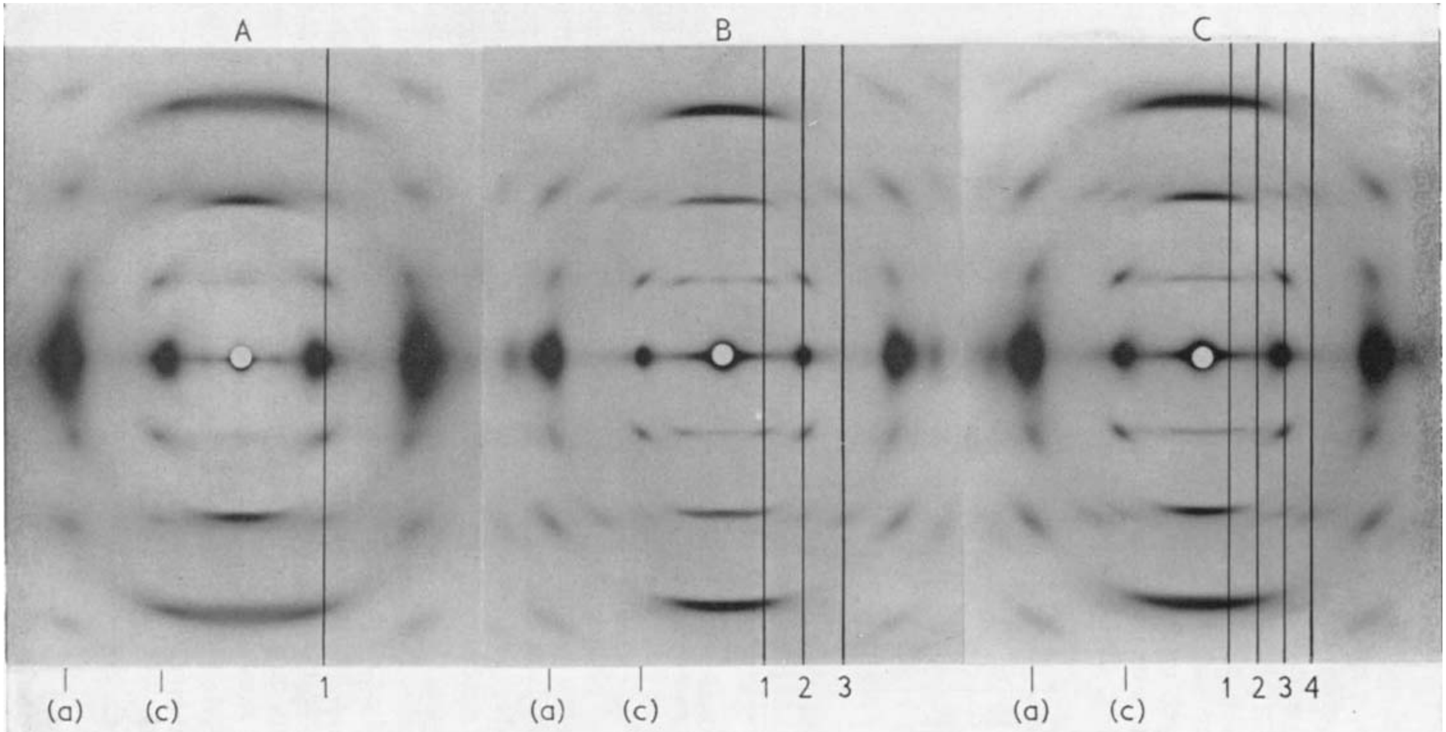

FIG. 1. X-ray photographs of chitins. Fibre axis vertical. Vacuum camera. A. β-chitin; B. α-chitin; C. γ-chitin. (The approximate positions of the row lines 1, 2, 3, etc. are indicated.)

in B and the third order in C. That is, along the c-axis of the structure the chains repeat in groups of one in A, in groups of two in B and in groups of three in C. This last form, C, is new and we propose to refer to it as γ-chitin.

Figure 1 B is the well known diffraction diagram of α-chitin, and Carlström (1957) was the first to give, by modern standards, an acceptable cell and space group. Along the c-axis neighbouring chains are alternately *up* and *down*. It follows, then, that in Fig. 1 A all the chains have the same direction, the c-axis period being only half that in Fig. 1 B. In Fig. 1 C the c-axis period is at least three times that in Fig. 1 A.

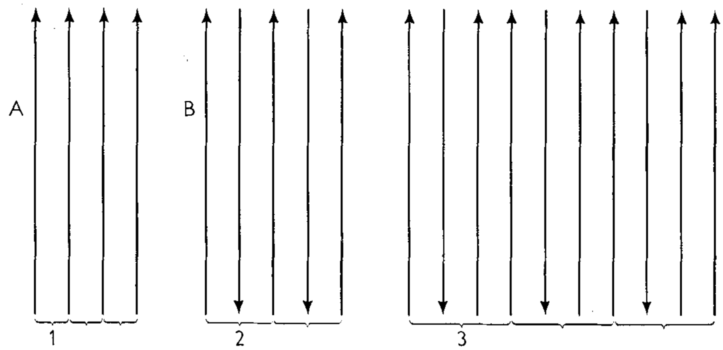

FIG. 2. Representing chitin chains viewed in the plane of the sugar rings. Chain arrangements in groups of 1, 2 and 3.

A very diagrammatic picture of our three types of chitin as projected on the ab plane is shown in Fig. 2, and the chain arrangement in groups of one, two and three along the c-axis is clearly seen. Parallel arrangement of chains as in 2A or antiparallel arrangement as in 2B are readily acceptable possibilities and are commonly met with in all sorts of fibrous structures. But arrangement of non-helical chains in groups of three is quite novel. The simplest form of chain grouped in sets of three would arise from the folding of a single chain upon itself as in Fig. 3, giving rise to three parallel segments. But we must justify the existence of bends in a chain molecule and, in particular, in a polysaccharide chain. The first clear example of very frequent folds joining lengthy straight segments of a naturally occurring chain molecule was shown in the cross-β structure of *Chrysopa* egg-stalk fibroin. Moreover, this cross-β configuration could be transformed to a parallel-β condition by highly stretching the silk-like fibres (Parker and Rudall, 1957). Regular folds are often met with in a wide variety of hydrocarbon chains and even when bulky side chains are present (Keller and O'Connor, 1958).

The first indication of folding of polysaccharide chains upon themselves came from a consideration of the structure of chitin itself. The β-form of chitin, Fig.1 A, is viewed as having all its chains "parallel", i.e. pointing in the same direction; experimentally it is a much less stable form than the α-structure of Fig.1 B. If β-chitin fibres are placed in 6N HCl, they contract to about half their length and the molecular

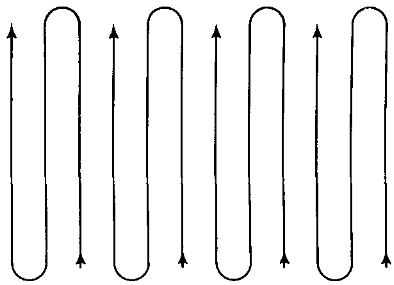

FIG. 3. Folding of a chain upon itself to give three parallel segments.

structure changes over to the α-type with antiparallel chains. As is seen in Fig. 4, we can readily achieve all these things by folding each chain upon itself, i.e. 50% contraction and change from β to α structure. The above change occurs in the swollen but solid fibrous state and does not involve a stage where the chitin is in solution. If, however, the

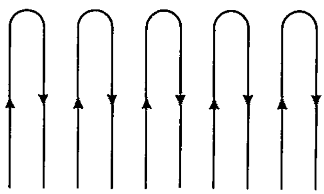

FIG. 4. Chitin chain contracted to about half its length by folding upon itself.

β-chitin is dissolved, say, in formic acid, the chains on precipitation recrystallize in the more stable α-form. The phenomenon of contraction in β-chitins with the development of α-chitin structure requires much further study, and it is particularly connected with the details of inter-chain reactions.

Cellulose-type chains are described as probably folding on themselves in Manley's study of crystals of cellulose tri-acetate and of cellulose II derived from these crystals. The data given is insufficient to tell us the thickness of the laminae in the crystals; we have to assume

from the context that this is much less than the chain length of about 1500Å (Manley, 1961). Similar evidence comes from a study of the morphology of single crystals of xylan (poly-β-D-[1$\rightarrow$4] anhydroxylose). Here the crystals show numerous thin laminae of about 50Å thickness, yet the known chain length is about three times this thickness (Marchessault *et al.*, 1961). The evidence clearly points to the conclusion that chains of 1$\rightarrow$4 β-linked pyranose residues can fold upon themselves and still yield "good" crystal structures as seen in X-ray diffraction diagrams.

We approach a more detailed consideration of the results of X-ray analyses of chitins with the concept that in the solid state the chains may be parallel or antiparallel or folded; we can conceive quite a variety of different secondary structures depending on the details of the folding.

a. The structure of α-chitin. Three considerable studies have been made in an effort to determine the structure of α-chitin by X-ray analysis. The pioneer work by Meyer and Pankow (1935) told us that the fibre axis repeat was almost identical with that of cellulose, and thus in the two polysaccharides there must be the same β 1$\rightarrow$4 link between pyranose rings which form straight chains. The dimensions given for the lateral separation of the chains were also essentially correct. But their consideration of $h00$ reflections was inadequate and led to an incorrect cell and to the presentation of models (often reproduced by others) in which the chain molecules are incorrectly placed. At the time, the importance of interchain bonding was scarcely recognized and there were no infra-red absorption data to say that certain interchain bonds must be allowed for.

More than twenty years elapsed before Carlström (1957) re-examined the structure of α-chitin using many modern resources. By reconsidering the indexing of some weaker reflections, he proposed a simpler orthorhombic cell with only half the dimensions of Meyer and Pankow's cell along the a-axis. Eliminating one possible type on stereochemical grounds, he arrived at an orthorhombic cell with: $a = 4{\cdot}76$Å; $b = 10{\cdot}28$Å; $c = 18{\cdot}85$Å; and of space group $P2_12_12_1$. This states that there are two chains running through each cell and that these chains are antiparallel. The particular relative intensities of the various reflections in the X-ray diffraction pattern depend upon the exact relative positions of the chitin chains and, of course, on the detailed configuration of the main chain itself and of its side chains. Carlström made numerous optical diffractograms varying such features as slight chain rotation about its axis and several positions where a chain is moved along its axis relative to the position of the

neighbouring antiparallel chain. By such trial and error methods, he reached the "best fit" between the optical diffractogram and the X-ray diffraction pattern; the composite diffractogram published shows impressive agreement with the X-ray diffraction photograph (Carlström, 1957).

There are many implications of Carlström's structure where it gives information on intra- and interchain bonding. The dominant and fixed feature of chain association is in the formation of hydrogen bonds of the type N—H . . . O═C between the aminoacetyl side chains; there

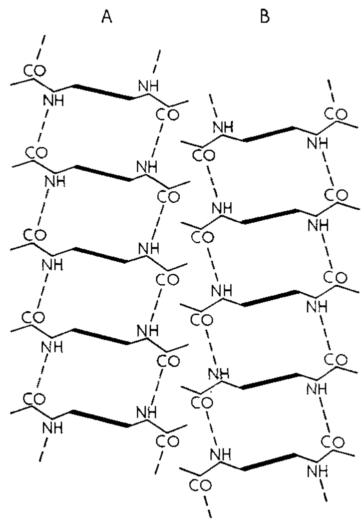

FIG. 5. Showing two piles of chitin chains A and B linked through C═O . . . HN groups. Chains "up" in A and "down" in B, as in α-chitin.

is full infra-red absorption evidence for such bonds which show high perpendicular dichroism. This special structural feature of chitin is indicated in Fig. 5 and consists of a pile of chain A linked through C═O and NH groups and seen here looking down the length of the chains which all have the same direction. α-Chitin is built up of two piles of chains A and B, the chains having opposite direction in the two piles. The fixing of the position of the NH . . . O═C bond is a highly satisfactory feature, but there still remain uncertainties about the bonding of OH groups in the structure.

Independently, Dweltz had been making a similar X-ray study, again on lobster tendon. He arrived at the same orthorhombic cell of space

group $P2_12_12_1$ but with slightly different dimensions: $a = 4{\cdot}69$Å; $b = 19{\cdot}13$Å; $c = 10{\cdot}43$Å. (He is reversing the assignment of axes b and c as used by Carlström, Meyer and Pankow and by others.) Dweltz (1960) uses a very mild purification, treating the tendons in cold dilute caustic potash and then in dilute hydrochloric acid, about half an hour in each, followed by washing. But this does not seem likely to be the source of the larger b and c dimensions. Dweltz gives no experimental error for his cell dimensions: his data were obtained from microcamera photographs and, as far as the fibre axis period is concerned, only four layer lines were considered. By contrast, Carlström used a cylindrical camera of 11·4 cm diameter and made measurements up to the eighth layer line. On these grounds the accuracy of Carlström's measurements should be much greater.

Another difficulty is that Carlström and Dweltz arrive at significantly different structures which are both said to give calculated intensities or relative intensities in experimental diffractograms, which agree reasonably well with those of X-ray diffraction photographs. If this is so, then there should be still other structures giving equally good or even, perhaps, better agreement. However, it seems that mistakes have been made in the derivation of one of these structures. Carlström (1962) successfully defends his structure by demonstrating the support coming from a considerable analysis of crystalline cellobiose which agrees with details of the chain configuration which he adopted for chitin on stereo-chemical grounds. He also maintains that there are errors in the atomic co-ordinates listed by Dweltz and from which he has calculated the intensities.

However, the solid measure of agreement lies in the existence of linked piles of chains as in Fig. 5 and in finding the same space group. It seems that the "buckled" main chain of Carlström with intra-molecular hydrogen bond between O_3 and O_5' of the next residue is probably correct. (See Fig. 10.)

We are more likely to discover other things about chitin by a combined consideration of the X-ray data and the infra-red absorption data, together with studies of models. Little more can be done on the direct determination of structure unless we can find much more highly crystalline preparations.

b. The structure of β-chitin. The structure given above for α-chitin is only one of a number of possible arrangements of chitin chains. The real existence of two other arrangements is demonstrated (Rudall, 1962a). For some time it has been recognized that β-chitin should correspond to a structure with all chains parallel. That is, if α-chitin

can be represented by two piles of chains having opposite chain direction, as in Fig. 5, A and B, then β-chitin should be represented by two piles of chains A, A, Fig. 6, the chain direction being the same in each pile.

Early studies on β-chitin showed that water played a significant part in the crystal structure, Rudall (1955), and in a recent extensive analysis Dweltz (1961) places one water molecule per acetylglucosamine residue in his model. Also he finds a simple cell with only one chain passing through it so all the chains in a crystallite must have the same direction.

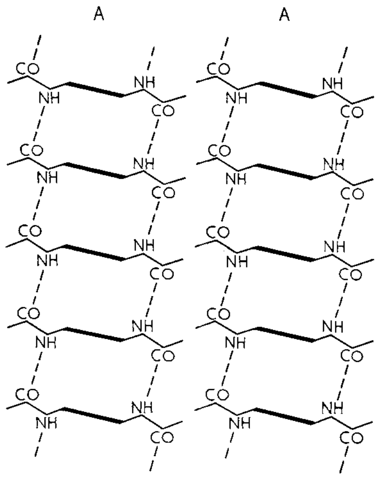

FIG. 6. Two piles of chitin chains A and A as is probable in β-chitin.

The cell is essentially rectangular. One has not sufficient confidence in any of the cell dimensions so far given for β-chitin to be able to say with certainty that the apparent fibre repeat is less in β-chitin than in α-chitin, as was first suggested by Lotmar and Picken (1950). If it is less it may even be that the cell tends to be triclinic as it is, for example, in nylon (Bunn and Garner, 1947).

It is likely that the detail of Dweltz's structure for β-chitin is unsatisfactory in that he keeps the same straight backbone as he used in his α-chitin structure; it seems to us that the criticisms of this by Carlström (1962) are justified. We would agree that there is a structure with one water molecule per residue and that the *Loligo* pen, left at room humidity, may well approximate to this monohydrate. Nevertheless, it is clear from the simple data already given (Rudall, 1955) that "equatorial" spacings and intensities alter significantly when the

diffraction pattern of β-chitin (*Aphrodite* chaetae) is obtained from thoroughly dried material maintained in a sealed dry cell.

At present the X-ray analyses are not providing any final conclusions about the packing and possible intermolecular bonding of chains along the c-axis in the case of β-chitin. But we feel confident that the chains are all parallel and that they have the same configuration as in α-chitin. A dominant fact about β-chitin is that water penetrates the crystalline structure freely and it is this feature which makes it a less stable structure than α-chitin.

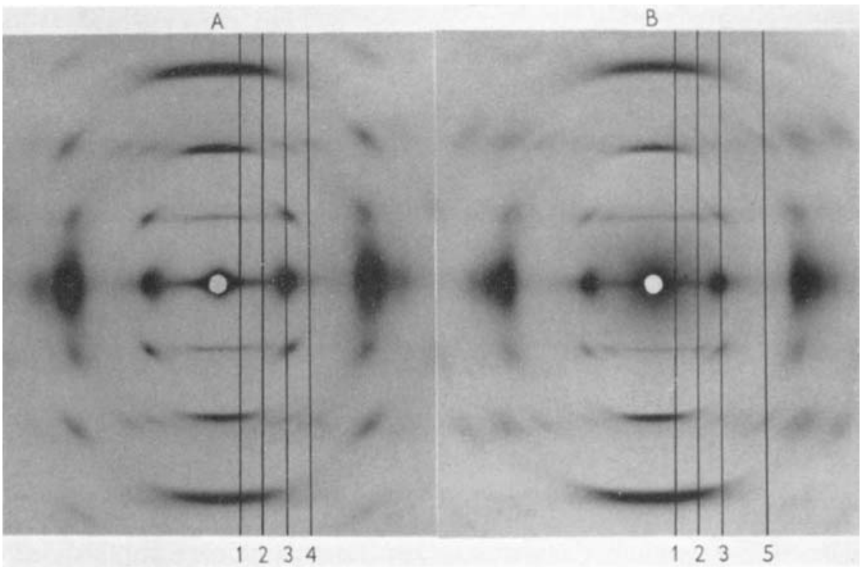

FIG. 7. X-ray photographs of fibrous γ-chitin. A. Dry in vacuum camera; B. At room humidity.

Further detailed studies of β-chitin are much more profitably made on the very highly crystalline β-chitins of Pogonophores. Here, three clearly different diffraction patterns have been obtained depending on the extent of dehydration. Infra-red absorption studies on these by Parker are important in going some way to resolve uncertainties about chitin. Indeed, the range of results for different types of β-chitin have been particularly illuminating and we will consider the infra-red results in a later section.

c. The structure of γ-chitin. We have spoken of the structure as having arrangement of chains in groups of three along the c-axis

according to Fig. 3. Thus we should have, in the one structure, inter-chain arrangements which are met with separately in α- and β-chitins. γ-Chitin would therefore be expected to form hydrates as does β-chitin. The air-dried and vacuum-dried forms of γ-chitin are shown side by side in Fig. 7. The position of row lines 1, 2, 3 and 5 are clearly evident in the air-dried form, Fig. 7B, while in the vacuum-dried material, Fig. 7A, row lines 1, 2, 3 and 4 are evident while row line 5 is very weak indeed. If the *a* dimension is *c*.4·7Å, then all these row lines must pass through *00l* positions on the equator. In the air-dry specimen, the reflection corresponding to 012 is obviously stronger than in the vacuum-dried material. These features indicate the kind of thing we must take into account in trying to define the exact position of water in the structure.

The great interest in γ-chitin lies in explaining why some adjacent piles of chains, like A and B in Fig. 5, and again some adjacent piles, like A and A in Fig. 6, occur together in the one structure so that there is grouping of piles of chains in sets of three. It is probably the details of the chitin/protein association which causes a folding of the chains as in Fig. 3.

2. *The shape of crystallites*

X-rays enable us to define the relative dimensions of the crystallites along *a*-, *b*- and *c*-axes; stretching, for example, causes the longest dimension to lie parallel to the direction of stretching, while pressing causes the thinnest dimension to be parallel to the direction of the applied pressure. In this way we find that the fibre axis of chain molecules, in their extended state, generally lies parallel to the longest dimensions of the crystallite. Is there any evidence that naturally occurring crystallites of chitin show considerable variety in their relative dimensions along *a*-, *b*- and *c*-axis?

The best known type, and that which was first clearly demonstrated in the case of soft insect cuticles (Fraenkel and Rudall, 1940, 1947), shows the thinnest dimension to be in the side-chain direction, i.e., along the *c*-axis, with crystallites being of considerably greater extent along the *a*-axis, but, of course, of far the greatest extent along the *b*- or fibre axis. The *b*-axis is the direction of covalent bonding, the *a*-axis is the direction of hydrogen bonding through NH . . . O=C groups while, as we will see later, there is probably only van der Waals' attraction along the *c*-axis. The size of the crystallites decreases along axes of weaker types of bonding.

In blowfly larval cuticles these relative dimensions of the chitin crystallite are shown by the dried, but otherwise intact, cuticle. But they

also persist in the purified structure after removal of protein by hot dilute caustic alkali or with diaphanol (Fraenkel and Rudall, 1940). By contrast, there seems to be no uniplanar or double orientation in the case of purified crab tendon chitin. That is, if one strongly compresses crab tendon there is no evidence for the *a*- or *c*-axes being preferentially oriented with respect to the direction of compression. Thus, in tendon, crystallites may be of comparable dimensions in the *a*- and *c*-direction, or if a distinct difference does, in fact, exist, it is not observable as a result of these compression experiments.

Marchessault *et al.* (1960) report on the shape of crystallites in partially hydrolysed chitin (presumably from the crab carapace) in a preparation which apparently removes amorphous fractions (Marchessault *et al.*, 1959). There is evidence that the crystallites are now considerably greater in the *c*-direction than in the *a*-direction. It seems unlikely that this is a natural condition, but rather that it has been produced by the treatment. Indeed, one would propose that blowfly cuticle chitin should be similarly refluxed in 2·5N HCl to see if the crystallites change from the natural state with the *a*-direction being greater than the *c*-direction, to a state similar to that in isolated crab crystallites where the *c*-direction has become greater.

According to Giles *et al.* (1958) refluxing in 2·5N HCl would be likely to swell the chitin greatly, and especially to alter the bonding of the structure along the *a*-direction. Ultrasonic treatment may then subdivide crystallites in this direction. If some permanent chemical change is also effected, and they have suggested removal of acetamido groups, it is likely that the form of the isolated crab crystallites is quite unlike that occurring in the natural state.

The above comments on the shape of crystallites refer only to α-chitin. No similar studies have been made on β- or γ-chitins. As regards orientation and relative dimensions, Heyn (1936) found the same type of crystallite in the apparent α-chitin of the cell wall in the fungus *Phycomyces* as is present in the blowfly larval cuticle. One should perhaps note that Clark and Smith (1936) observed no orientation of crystallites with the 002 planes lying parallel to the surface in the case of reprecipitated films of carapace chitin dissolved in strong hydrochloric acid. However, in their chitosan prepared from a sheet of chitin such as the lobster carapace, they observed that the 002 planes lie parallel to the surface. We have given elsewhere (Darmon and Rudall, 1950) some notes on crystallites in chitin nitrate. The product which we obtained by nitrating blowfly cuticles had the kind of orientation described above for crab crystallites isolated by boiling in 2·5N HCl.

We conclude that it is only the shape of the crystallite in the natural structure which may have special significance in relation to other constituents such as protein. In various purified or modified chitins the crystallites are probably considerably altered in shape and dimensions.

3. *Anomalous X-ray effects*

Besides effects due to white radiation, which are particularly obvious on the equator, there are some rather well-defined reflections which may have special structural interest.

a. Meridional reflections. In α- and β-chitins which have been purified by the standard method of 24 h in boiling 5 or 10% caustic alkali, there is usually an apparent reflection about the 010 position and this is often illustrated in X-ray diffraction photographs. (See Carlström, 1957; Dweltz, 1960; Rudall, 1962a; and Fig. 1A and 1B of this article.) Perhaps the most obvious of these 010 type reflections is that illustrated for a dried β-chitin (Rudall, 1955, Plate 3). It is very difficult to dismiss this as an insignificant part of the structure. But it is customary to discount these sometimes very obvious reflections in order to support an exact twofold screw axis in the fibre direction. This type of reflection remains of interest to us in connection with the problem of bound water in chitin and by the presence of some significant number of glucosamine residues.

In the diffraction diagram of γ-chitin shown in Fig. 1C, we have medium-strong near-meridional reflections which are tentatively indexed 011 with the *c*-axis period being near to 29Å. It is possible, with other more "folded" forms of chitin chain, to have essentially α-structure, but with a true *c*-axis period of greater value, say 50–100Å. In this case 011 reflections would be scarcely distinguishable from the forbidden 010 reflection.

We might record an experience which has not so far been followed up. During the drying of purified β-chitin (*Aphrodite* chaetae) in a small cell in the presence of P_2O_5, the fibres may have become contaminated with phosphoric acid during subsequent washing. Near the meridian in this probably phosphorylated chitin, Fig. 8, all layer lines 1, 2 and 3 are distinct and of comparable intensity. There are other obvious changes in the spectrum, but we are drawing attention to the well-defined reflection extending across the meridian on the first layer line. Here, too, it is probable that the near meridional intensity is due to 011 (or *01l*) reflections coming from an altered β-chitin structure following the treatment, in this case suspected phosphorylation.

The purified chitins often show some defined scattering at about the

010 position and this can vary in intensity according to the method of purification and other treatments. We will see later, in dealing with chitin/protein complexes, that similar reflections can be part of an elaborate meridional series.

b. Equatorial reflections. The contribution from "white" radiation is obviously very great on the equator and the significance of weaker reflections can only be assessed using monochromatic radiation. For example, the apparent equatorial reflection at about 23Å in β-chitin from *Aphrodite* and *Loligo*, as illustrated in Fig. 1A, is still observed using monochromatic radiation reflected from calcite.

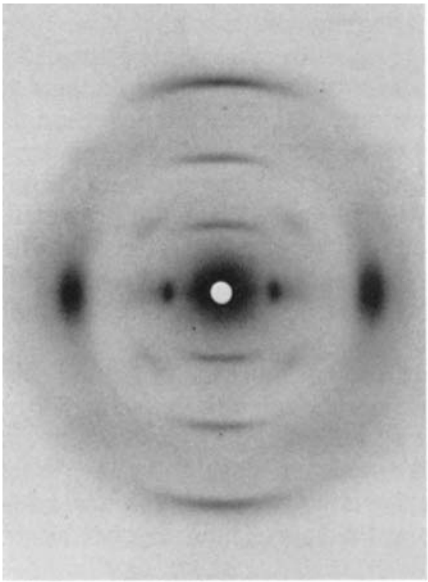

FIG. 8. Purified β-chitin of *Aphrodite* chaetae after suspected phosphorylation.

A series of reflections at smaller angles are mentioned by Clark and Smith (1936) in the case of chitosan fibres and also with addition compounds of chitosan. One is tempted to account for this well-defined series as being due to another component such as lipids. Nevertheless, it may be an error to ignore scattering information that does not conform to concepts of pure anhydrous polyacetylglucosamine constitution or perfect twofold screw axes.

Another type of equatorial reflection is usually observed in strong photographs of well-crystalline α-chitins; these very conditions suggest it is either due to "white" radiation or is perhaps part of a superlattice.

Studying these weaker reflections by monochromatic radiation would be very time consuming and here is clearly a case for establishing the necessary electron diffraction technique.

On the equator there are probably several reflections, the origin of which should be critically studied, but the one that is most generally obvious has a Bragg spacing of about 5·6Å. Indeed, this is referred to by Dweltz (1960) and is clearly visible in Fig. 2e published by Carlström (1957). We have often observed this apparent reflection in various α-chitins and also in β-chitin. These reflections may be of trivial origin, i.e. so-called radiation artifacts due to the non-monochromatic X-ray sources used. But our interest in them is based on the possibility of other linkages between chitin chains that may possibly occur under some

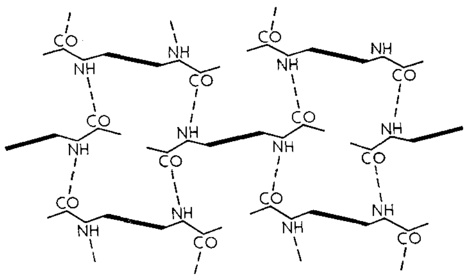

FIG. 9. A possible method of linking chitin chains through C=O . . . NH groups.

conditions, for example at the surface of crystallites. We suggested the type of linkage in Fig. 9 on a previous occasion (Darmon and Rudall, 1950), and such a structure seems possible if water molecules help to fill space between chains in the *c*-direction. Many reflections arising from an arrangement as in Fig. 9, would not be easily separated from the standard chitin patterns as Fig. 1, A, B and C, but one would expect a strong equatorial reflection in the region of 5·5Å; as we have pointed out above, there are weak reflections near that position which are still unaccounted for.

Some degree of experimental study on wet and dry chitins and using monochromatic radiation should provide definite explanations for these several anomalous effects.

C. INFRA-RED ABSORPTION

The first attempt to give assignments for the main absorption bands in chitin, chitosan and chitin nitrate was made by Darmon and Rudall (1950). They recognized the importance of NH . . . O=C bonds

between aminoacetyl groups of neighbouring chains, but were surely wrong in proposing that only half the aminoacetyl groups were bonded in this way. Carlström (1957) corrected this by finding perhaps the true unit cell and proposing that all NH groups were hydrogen bonded to C=O groups in neighbouring parallel chains. Yet, in detail, we do not agree with the spectra presented by Carlström and published again by Engström and Finnean (1958). We maintain that there has been an error in drawing out the spectra in their case and that the dichroisms they dispute were, in fact, correctly indicated by us. We are supported in this by Pearson *et al.* (1960), who find the same dichroisms in the region 1200–1500 cm^{-1}. However, for our present discussion such absorption bands are not very crucially involved in the interpretation of structure. But there are five main absorption bands which are most important in this regard, namely the bands at 3485 and 3445 cm^{-1} attributed to O–H stretching frequencies, the so-called second NH stretching band at 3100 cm^{-1} and the two rather mysterious bands at 1656 and 1625 cm^{-1}.

1. *N–H stretching vibrations*

Of the above five bands that at 3100 cm^{-1} is most readily accounted for. Dweltz (1960) tried to use this as an O–H stretching frequency with perpendicular dichroism, which he needed in order to explain hydrogen bonding between hydroxymethyl groups in the lateral direction. Experimentally the absorption at 3100 cm^{-1} is still present when OH groups have been substituted by nitration (Darmon and Rudall, 1950). The 3100 cm^{-1} band is now recognized as resulting from Fermi resonance between the overtone of the NH deformation vibration at about 1550 cm^{-1} and the main NH stretching frequency at about 3250 cm^{-1} (Cannon). Parker has made some very neat studies of the origin of this band in the case of various chitins and the assignment to a hydrogen-bonded NH vibration seems the only reasonable one.

Pearson *et al.* (1960) likewise do not accept Dweltz's suggestion that the 3100 cm^{-1} band indicates an OH stretching vibration of perpendicular dichroism. They consider there is no evidence for OH stretching vibrations in chitin, except those having a parallel dichroism.

2. *O–H stretching vibrations*

The two bands at 3485 and 3445 cm^{-1} are of this type. They are extensively altered by deuteration. In chitin there are two OH groups, that on C_3 and that on C_6 (Fig. 10A) and two different types of OH bonding are to be expected. Carlström (1957) proposed a hydrogen

bond between O_3 and O_5' of the next residue (Fig. 10B), and this seems very acceptable on stereo-chemical grounds; it is found in crystalline sugars, crystalline cellobiose and is likely to occur in cellulose itself (Liang and Marchessault, 1959). These authors regard the strong absorption at 3350 cm^{-1} in native cellulose as due to this type of

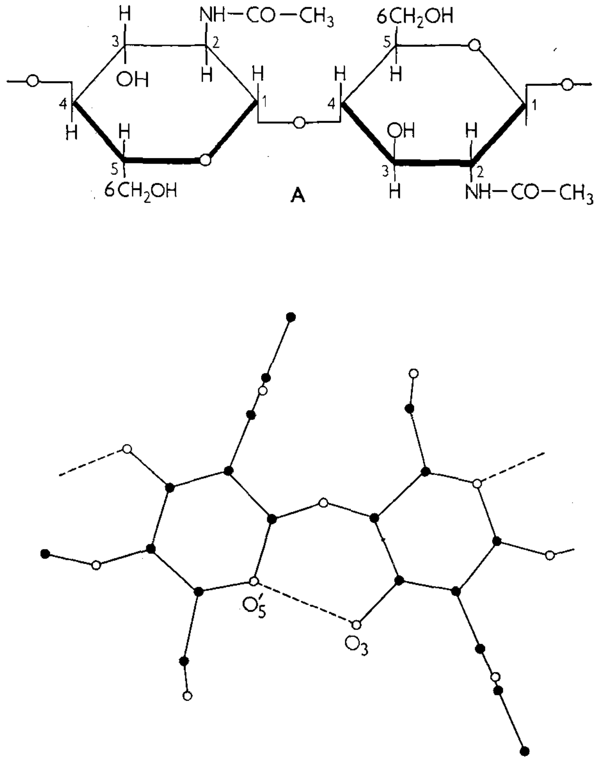

FIG. 10. A. Structural formula of chitin; B. Intramolecular bonds in chitin.

intramolecular band. In chitin the same band should be associated with the absorption at 3445 cm^{-1}.

We should note in passing that the same two prominent bands in chitin at 3485 and 3445 cm^{-1} are also found in mercerized cellulose, i.e. cellulose II, and have similar strong parallel dichroism. Marchessault and Liang (1960) favour the interpretation that both are due to intramolecular bonds of the type O_3–O_5' (See Fig. 10B).

Parker finds a number of details within this region of the spectrum in different chitins and in relation to varying effects following deuteration and on drying. The more constant band is that about 3445 cm^{-1}; the band at 3485 cm^{-1} is often much weaker, or even effectively absent in highly crystalline forms of β-chitin. We might note that in the progressive deacetylation of α-chitin the band at 3485 cm^{-1} is disappearing, while that at 3445 cm^{-1} remains; this is associated with the persisting "backbone" of the polysaccharide chain.

Apart from knowing that they arise from hydrogen-bonded OH groups and that they have parallel dichroism, the more detailed origin of the absorptions is not well understood.

3. *The carbonyl absorption*

The most interesting new information which Parker has obtained in his study of chitins concerns the interpretation of the two bands at 1656 and 1625 cm^{-1}. Darmon and Rudall (1950) regarded these as both being carbonyl frequencies and preferred to look on the 1656 cm^{-1} band as being typical of that associated with C=O . . . NH bonding as in amides, polyamides and proteins. The band at 1625 cm^{-1} seemed to be lost during progressive deacetylation and during nitration.

Pearson *et al.* (1960) also consider their band at 1652 cm^{-1} to be the main C=O stretching frequency, or amide I band, but give two suggestions for the origin of the absorption at 1619 cm^{-1}. One involves a C=N stretching mode of an enol form and the other a C=O stretching mode of a rotational isomer of the aminoacetyl groups (Marchessault *et al.*, 1960).

These interpretations have all been made by comparison with reported absorptions of amide groups in various compounds. Parker's observations on the deuteration of α- and β-chitins suggest quite a different interpretation. Normally α-chitin, when treated in D_2O, shows no change in the 1656 cm^{-1} band, as is also found by Marchessault *et al.* (1960). But the same band in β-chitin is readily removed by deuteration, the band at 1625 cm^{-1} remaining. If one purifies intact tendon chitin in NaOD, then the band at 1656 cm^{-1} is considerably weakened as if it were partly moved by deuteration. Some samples of apparently α-chitin structure according to the X-ray diagram, such as the purified proximal tip of the radula of *Patella* (Runham, 1961b), also behave like a β-chitin and the 1656 cm^{-1} band is removed on exposure to D_2O vapour. These experiments then suggest the following quite new interpretation. The band at 1625 cm^{-1} is the real C=O stretching absorption, while the band at about 1650 cm^{-1} is due to bound water. Two of Parker's

spectra are shown in Fig. 11, A illustrating the weakening of the band at 1656 cm^{-1} in the case of crab tendon, and B the disappearance of this band from proximal *Patella* radula chitin following deuteration.

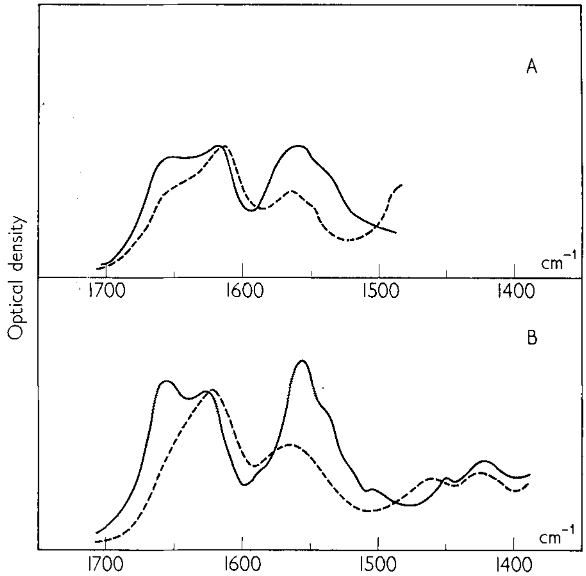

FIG. 11. Infra-red absorption spectra of chitins (Parker). A. Blowfly larval cuticle: purified in NaOD, ---; purified in NaOH, ——. B. Purified proximal region of Patella radula: in air, ——; in D_2O vapour, ---.

D. A NEW VIEW OF THE STRUCTURE OF α-CHITIN

X-ray analysis showed that water is normally a part of the crystalline structures in β-chitin and several different hydrates can be formed at will, especially in Pogonophore tubes. The finding that the band at 1650 cm^{-1} can be readily moved by deuteration indicates that this is due to bound water. The same band in most α-chitins, such as crab tendon and blowfly cuticle, is very difficult to affect by deuteration, but it can be considerably altered by deuterating during the removal of protein, that is, when the chitin chains are less aggregated. Thus in α-chitin, too, the band at 1650 cm^{-1} can be assigned to the presence of bound water.

If one assumed there was one water molecule per *N*-acetylglucosamine residue in the standard α-chitin of, say, lobster or crab tendons, then using the X-ray dimensions of Carlström, the density would be 1·58. This is the value expected in cellulose, but is very much higher than that

recorded for α-chitin, namely 1·4–1·425 g cm^{-3}. Or, if we supposed one water molecule per chitobiose unit, then the density is still unreasonably high, 1·52, compared with the measured values.

In the case of β-chitin there is no special difficulty. Taking reasonable X-ray values of a = 4·7, b = 10·28 and c = 10·5Å and with one water molecule per *N*-acetylglucosamine residue, the density is calculated to be 1·44 g cm^{-3} which is in fair agreement with the measurements of Lotmar and Picken (1950), namely 1·40–1·42.

Another possible way of accounting for bound or trapped water is in connection with the presence of a certain number of deacetylated residues, that is, glucosamine residues: if each acetyl group was replaced by two water molecules, the density of the structure would remain essentially the same as for poly-*N*-acetylglucosamine.

In this way we could account for a number of things at once. The evidence from analysis of elements by Giles *et al.* (1958) proposed a constitution of chitin with about one in six or seven of the acetylglucosamine residues being deacetylated and with a significant amount of bound water. The infra-red absorption data suggest considerable bound water which is normally inaccessible to deuteration. But the X-ray and density measurements say that, in mass, the chain structure must be the equivalent of poly-*N*-acetylglucosamine. The nearest way of satisfying all these requirements is to replace some significant number of acetyl groups by water molecules.

It is right to propose these things which are in reasonable agreement with the known or apparent facts. The crucial argument rests on the interpretation of the infra-red absorption bands at 1656 and 1625 cm^{-1}. Studies on *N*-acetyl chitohexaose by Pearson *et al.* (1960) state that the crystal structure is only slightly different from that of chitin itself, while the infra-red absorption spectrum they publish indicates the same two prominent bands near 1625 and 1650 cm^{-1} but both are apparently somewhat shifted to higher frequencies. On the other hand, *N*-acetylated chitoheptaose and chitin are said to give the same X-ray diagram and the same infra-red spectrum and, indeed, a density value of 1·424 was obtained for the chitoheptaose (Marchessault *et al.*, 1960). Here we cannot suggest "deacetylation and bound water" as the explanation of the strong absorption at 1650 cm^{-1}. But in view of Parker's infra-red studies on α- and β-chitins, the behaviour of the chitohexaose and -heptaose in the presence of D_2O or on extensive drying should be closely examined. The question is, does the band near 1650 cm^{-1} in these chitin oligosaccharides arise from the presence of water in the structure?

Purified chitins certainly vary in the sharpness and perfection of the

X-ray diagram and this could possibly be related to the presence of varying numbers of residues which are glucosamine. Some examples of less perfect X-ray diagrams as a result of treating chitin in cold concentrated HCl have been given by Clark and Smith (1936) and, among other things, they proposed that some of the decreasing "crystallinity" is due to loss of acetyl groups. One would expect the lobster carapace of Clark and Smith to be essentially the same as that used by Giles *et al.* (1958) and that the X-ray diagrams presented were those for a polysaccharide containing about 80% of *N*-acetylglucosamine residues if one accepts the interpretation of chemical analysis as given by Giles *et al.*

We have purposely tried to see what case could be made out for a significantly new structure for α-chitin. However, we might conclude by a more conservative view. Chitin as a polysaccharide may not be truly poly-*N*-acetylglucosamine, but the suggestion of other than rather small quantities of glucosamine may be unwarranted. The X-ray diagram and the density correspond to anhydrous poly-*N*-acetylglucosamine. The infra-red spectrum of chitin and of established *N*-acetylheptaose have the same characteristic absorption bands near 1656 and 1625 cm^{-1}. The only sound reason for suggesting that we might have to move from this conservative position lies in some unusual properties associated with these bands.

III. THE CHEMISTRY OF THE PROTEINS ASSOCIATED WITH CHITIN

In our first view of cuticle composition (Fraenkel and Rudall, 1947), we tried to find the kind of relationship between the chitin and the protein. We argued that the maximum chitin content of unaltered cuticle substance corresponded very nearly to a system with an equal number of chitin and β-protein chains of the same length, indicating that there was, perhaps, some structural significance in parallel and associated chains of equal length. Most of this protein could be removed by treatment in boiling water leaving a tough aggregated residue which gave the X-ray diagram of purified chitin. There is now much more information about the proteins occurring with chitin in cuticles and other structures, and it helps in the process of forming a picture of chitin/protein structures at the molecular level.

A. PROTEIN FRACTIONS FROM "SOFT" INSECT CUTICLES

The water-soluble proteins of blowfly larval cuticle, lepidopteran larval cuticle and intersegmental cuticle of lobsters showed some rather

unusual properties. They were not coagulated by heat and were still soluble in hot trichloracetic acid. They were precipitated by aqueous solution of ethanol and by one third saturated ammonium sulphate. In many ways these were the properties of gelatin, yet the X-ray diffraction diagram of the insect cuticular protein was not comparable with that of collagen, but was like that of β-proteins (Fraenkel and Rudall, 1940; Trim, 1941). As is described by Richards and Pipa (1958) birefringent fibres can be drawn out of concentrated solutions of the cuticle protein. This property is typical of some other proteins in insects, namely the various silks; there may be interesting relationships between all these different epidermal proteins (Rudall, 1962b).

These same water-soluble proteins were studied in more detail by Hackman (1953) who found them to be heterogeneous in that they separated into three major components in electrophoresis. The largest component was found to have a different amino acid composition from that of the whole protein extract and it is probable that the other electrophoretically separated components have characteristic amino acid contents. But the whole water-soluble cuticular proteins of seven different insects which were examined were remarkably similar in their physical and chemical properties. We had called this mixture of proteins "arthropodin" (Fraenkel and Rudall, 1947), in the belief that there was a special protein present in all arthropod cuticles. Now there seems to be perhaps a characteristic mixture which is widely present; the centre of interest lies in associating special properties of the cuticle with variations in the mixture, which we called "arthropodin".

Hackman and Goldberg (1958) throw much further light on the proteins of insect cuticle by subdividing them into classes according to the conditions under which they can be extracted. For this purpose they worked on the huge larva of a beetle, *Agrianome spinacollis*, thus obtaining abundant soft cuticle which was relatively convenient to handle and to separate from other body tissues. For a soft cuticle this has a very high protein content, namely 63% protein: 37% chitin, the nitrogen content of the whole cuticle being given as 11·7%. Other soft larval cuticles have nitrogen contents ranging from 9–11·3% (Trim, 1941), but these may be too low because of the probable loss of protein in the procedure used for cleaning the cuticles. In the case of the blowfly larval cuticle *Sarcophaga falculata* (now *barbata*, Day, 1946) the nitrogen content is only 10·1% and the chitin fraction is 50% (Fraenkel and Rudall, 1947).

The main result of Hackman and Goldberg's study was the definition of five fractions which are extracted one after the other. In Table I

these fractions are listed as a percentage of the total protein and in parenthesis the fractions are listed as approximate percentages of the whole cuticle.

TABLE I

Definable protein fractions in a soft insect cuticle

A soluble in cold water	14%	(8)
B soluble in salt solution	2%	(1)
C soluble in 7M aqueous urea	25%	(16)
D soluble in cold dilute aqueous NaOH	3%	(2)
E remaining protein held more firmly	56%	(35)

The firmly held protein E is about equal in quantity to the chitin content (37%) of the cuticle. In connection with chitin/protein complexes such major fractions as C and E, which are closely bonded in the cuticle structure, are of greatest significance.

An obvious question of interest would be how the "spectrum" of fractions would appear in the *Sarcophaga* larval cuticle with its ratio of chitin/protein of about 50:45. It is clear that in such cuticles much of the protein is less firmly bound than fraction E of Table I, so that the equivalent fraction E of the blowfly larva would appear to be considerably less than the amount of chitin in the cuticle.

Again, it is of interest to compare the results in Table I with previous studies on water-soluble proteins from various cuticles by Hackman (1953). Such protein, which in the case of soft beetle larva cuticles amounted to about 24% of the cuticle, were extracted with buffers at pH 9·2, there being two extractions for 36 h at 50°C. To a first approximation these extracts would correspond to the combined fractions A, B and C of Table I.

Electrophoretically none of these various fractions appears homogeneous; the amino acid constitution is different in the several fractions of Table I. Hackman (1959) mentions a thesis by Moorefield (1953) reporting that "in the ultra-centrifuge the water-soluble protein behaves as a monodisperse substance and has a molecular weight in the region of 7000–8000 as determined from sedimentation constants". If this were true we would be led to the picture of several proteins of the same molecular size but of differing amino acid composition.

Because of Hackman's series of studies, we can see a rather detailed way of comparing different chitin/protein containing systems. It is to divide the protein into the various fractions shown in Table I, thereby displaying the relative proportion of each; the amino acid composition

and other properties can be determined for each fraction. A standard procedure is necessary if comparable results by different workers are to be obtained, and this applies particularly to the preparation of uncontaminated whole cuticle. Hackman's procedure is essentially the same as, and is indeed based on that of Fraenkel and Rudall (1947); thus the different ratios of chitin/protein found in *Agrianome* and in *Sarcophaga* larval cuticles should be a real difference.

The meaning to be attached to the various fractions is not very certain. Does the large water extractable fraction occur in the really native state? The cuticles have been immersed in 65% ethanol during cleaning, they have been ground to a fine powder with sand and then extracted in water for 72 h. These processes may alter configuration of proteins or remove small linking ions or other linking molecules. Some cuticles swell very markedly in water and this may involve irreversible changes. The various treatments, perhaps in particular the drying, may cause denaturation of proteins so that they remain unextracted. Silk fibroin-like proteins would not normally be soluble in 7 moles urea. Hackman and Goldberg mention some of these difficulties. Though our comments make interpretation of the results in Table I more complex, we think they are necessary.

B. CHITIN—A GLYCOPROTEIN

The possibility of a covalent link between chitin and protein chains has been considered for a long time. Foster and Hackman (1957) developed a method which appeared to isolate a chitin/protein entity. From the cuticle of the edible crab treated in ethylenediamine tetra-acetic acid at pH 9 and then at pH 3, they obtained a product with about 5% protein content. But when this product was dispersed in saturated aqueous lithium thiocyanate, the various fractions precipitated by acetone from the syrupy liquid still consisted of chitin and protein. This is not convincing proof that chitin and protein are definitely joined together, but it certainly suggests it.

In a considerable development of this approach Hackman (1960) isolates a similar chitin/protein complex from five chitinous tissues, using in each case EDTA or some other non-proteolytic procedure. The chitin/protein content of the product varies from 1:1 to 20:1. One is interested in the meaning of these ratios. Is there possibly a constant ratio of chitin/protein of 20:1 while in some cases additional protein is trapped in the solid residues? In many of these cases Hackman dispersed the products in lithium thiocyanate and examined the composition of the acetone precipitates from the syrupy solution. He

records that "the amino acid composition of each fraction appeared to be similar to that from which it was prepared", and that "in no case was a protein-free chitin obtained". But we do not know if a considerable portion of the protein is sometimes lost because it is not firmly attached to the complex.

The most intriguing conclusion from this work by Hackman concerns the last amino acids to remain with the chitin during hydrolysis. Starting with the chitin/protein product, of the beetle larva described in fraction E, Table I, consisting of equal quantities of chitin and protein, there is only some 5% protein after treatment in the proteolytic enzyme, papain. But when chitin/protein complexes are treated with *N* aqueous sodium hydroxide at 100°C (60 h for example) all protein seems to be removed except for the amino acids actually linked to the polysaccharide. The acid hydrolysis of these apparently well-purified chitins yielded glucosamine and always some amino acids, but only aspartic acid and histidine. This was always so whether it was crustacean or insect cuticle chitin, or molluscan chitins from squid, cuttlefish or nautiloid. It is most pleasing to learn that aspartic acid and histidine are apparently the connecting links between chitin and protein. In giving us some indication of quantities, Hackman (1960) says, p. 571, "There are two histidine and one aspartic acid residue for each 400 glucosamine residues", yet on p. 577 he gives another estimate of "one aminoacid residue per 200–300 glucosamine residues". There is, of course, the difficulty of estimating the amount of distruction of the glucosamine during hydrolysis but 200 residues would mean a polysaccharide chain about 1000Å long. This is within the range of length of natural fibre forming chain molecules, e.g. tropocollagen 2800Å and fibrinogen 500Å. The ratio of histidine/aspartic acid residues is estimated as 2:1. No special significance can be seen in this, apart from noting that a chain has two ends and one middle as in Fig. 4; and there could be other less resistent links which have been hydrolysed.

The finding of this constant presence of the above two amino acids, in conditions where all protein peptide links have been broken, allows the conclusion that protein chains are covalently linked to chitin chains and that the complex is a glycoprotein.

C. LINKAGES BETWEEN CARBOHYDRATE AND PROTEIN

We have inherited from the past a number of terms describing apparent complexes between protein and carbohydrate. Such terms as mucopolysaccharide, mucoprotein and glycoprotein are frequently met

with and often have no precise meaning. Where we are concerned with a stable covalent link between protein and prosthetic sugar groups, perhaps as disaccharide or highly polymerized chain, then the general term glycoprotein would seem to serve well; it is preferred by Hackman (1960) for what he calls "native chitin". This is the chitin chain together with its attached protein chain or chains; chitin itself, though ideally poly-*N*-acetylglucosamine, is, in practice, mostly *N*-acetylglucosamine with some glucosamine and some amino acids, e.g. histidine and aspartic acid. Mucoprotein and mucopolysaccharide are too suggestive of mucus secretions and gum-like substances; chitin differs so markedly from these in appearance and general properties that it is perhaps natural to dismiss them as inappropriate terms. Yet concerning the nature of the link between carbohydrate and protein we can learn something from the study of many substances whether they be called mucopolysaccharide, mucoprotein or glycoprotein.

Gottschalk *et al.* (1962) make the generalization that Nature always adheres to the glycosidic linkage when joining sugars to sugars, and to the amide linkage when joining amino acids to amino acids. "Yet in the attachment of carbohydrate to peptide almost any of the functional groups of the latter may be involved, resulting in a variety of modes of linkage." Presumably any functional group in the carbohydrate would likewise be involved.

But nearer to the possible situation in our chitin glycoprotein are some studies of ovine submaxillary gland mucoprotein—OSM. Gottschalk (1960) illustrates a glycosidic ester linkage between an *N*-acetylated hexosamine and the γ-carboxyl of a glutamyl residue in protein; or it could be linked with the β-carboxyl of aspartyl. In γ-globulin the link is said to be between aspartic acid and glucosamine; in ovalbumin linkage of carbohydrate to aspartic acid is said to be proved (see Gottschalk *et al.*, 1962). Thus in chitin, OSM, γ-globulin and ovalbumin aspartic acid is established as a link between protein and carbohydrate and it is probably linked to hexosamine in each case.

But what of the varying stability of these links. In OSM, Gottschalk *et al.* concluded that the disaccharide–peptide linkage was of the ester type and susceptible to hydrolysis in mild alkali; they cite other evidence that the glycosidic-ester linkage is sensitive to very mild alkali. But they contrast this with the relative stability of the *N*-glycosidic linkage to the amide group of asparagine. Still other types of linkage are: an amide link between a carboxyl of muramic acid and the amino group of a terminal L-alanyl unit; *O*-glycosidic linkage to serine.

On the grounds of the great stability of the link between the residual

amino acids and chitin, Hackman (1960) preferred the type of linkage *N*-acylglucosamine, i.e. linkage via a carboxyl to the NH_2 group of glucosamine. He supposed that this might have the stability of the *N*-acetyl link in chitin although the acetyl group in *N*-acetyl-D-glucosamine is not very stable.

Two things are apparent. How far are we justified in comparing the stability of a link to a large molecule with a supposedly similar link in a small model compound? Is, say, a supposed *N*-acylglucosamine link protected from hydrolysis by being included in the well-bonded system of chitin chains just as the acetyl groups are relatively protected in the crystalline chitin. With these things in mind we might ask if aspartic acid and histidine linked in exposed positions not within "crystallites" are lost during hydrolysis. The second important question is, "What other covalent links might occur in the chitin/protein complex involving other amino acids, but which we are overlooking?" At present the only ones we locate are those that survive 2½ days boiling in *N*-aqueous sodium hydroxide. There is a good case for pressing on with the use of proleolytic enzymes in an attempt to find other possible linkages between amino acids and chitin.

IV. X-Ray Studies on Chitin/Protein Complexes

With few exceptions, X-ray diffraction diagrams of chitinous cuticles are very different from the diagram of purified chitin. It was this modified chitin lattice that we considered to represent a chitin/protein complex (Fraenkel and Rudall, 1940, 1947). Since those first studies we have found a considerable variety of types of chitin/protein diffraction diagrams.

We can scarcely hope to interpret these patterns unless we get the maximum amount of detail in them; this is possible only in the most highly oriented fibrous specimens. In the case of soft larval cuticle or soft intersegmental cuticle from adults this involves stretching the cuticle to the maximum extent and under conditions that do not lead to "denaturation" of the protein. By "denaturation" we mean, in these cases, displacement of the protein or change in its configuration. Smaller changes in the protein, such as reaction with natural "tanning" molecules, may not prevent us from obtaining useful information about the structure of the complex.

In the case of hardened cuticles we take advantage of the fact that the molecular structure is often highly oriented as in a fibre. This is particularly so in long leg segments and in the veins of the wing. So in

a search for the most perfect specimens we look for these and, where possible, for the long fibrous or rod-like ovipositors which sometimes occur. In this way we can reach the conclusion that a particular detail is common to a whole order, or reaches its greatest expression in, perhaps, just one family. Having satisfactorily defined such a structure in a leg segment it is usually possible to detect it also in the less well-oriented material of tergites and sclerites.

From our studies of various insect cuticles during the last twenty years we can select examples which are particularly well defined. By continued selection of the most perfect specimens among these it would seem possible to establish some principal features of chitin/protein complexes.

A. SOFT LARVAL CUTICLES IN DIPTERA, LEPIDOPTERA AND COLEOPTERA

By far the greater number of our observations have been made on the larval cuticles of cyclorrhaphous flies as represented by the larger blowflies *Calliphora* and *Sarcophaga*. One can stretch the well-scraped cuticle of the larva in the fresh moist condition to give extensions of 30–40%. A moderately high degree of orientation of the fibrous structure is achieved and there is no tendency for the cuticle to contract on removing the tension. The orientation is achieved by slipping or drafting the submicroscopic fibrils by a process of plastic flow rather than by elastic deformation.

X-ray diffraction photographs of dried oriented cuticle and of the same cuticle with the protein removed, enable us to define the main features which are associated with this type of chitin/protein complex. These are illustrated in Fig. 12A and B for stretched lobster inter-segmental cuticle which gives essentially the same diagram as the soft cuticles of the dipteran larva. A is of the intact cuticle; B is of the cuticle after removal of the protein and it corresponds to a purified chitin.

In B the X-ray reflections are all comparatively sharp and well defined; the chitin shows about the same high degree of crystallinity as is found in purified tendons of lobsters and crabs. By comparison, the reflections in A are broad and ill defined except along the meridian or fibre axis. But laterally the chitin chain molecules have not aggregated into large crystallites, and apparently are prevented from doing this by their intimate association with protein.

The second main difference between A and B is the very strong

reflection at about 33Å on the equator of A, and the absence of this in B although there is a considerable "white" radiation streak on the equator there. The 33Å reflection in A moves to about 45Å on wetting in water. The third difference between A and B lies in the reflecttion at about the 010 position in A while this is absent or extremely weak in B.

A tentative interpretation of these general features is as follows. There is a layer of protein between every six piles of chitin chains ABABAB of the type shown in Fig. 5, giving a period of about 66Å

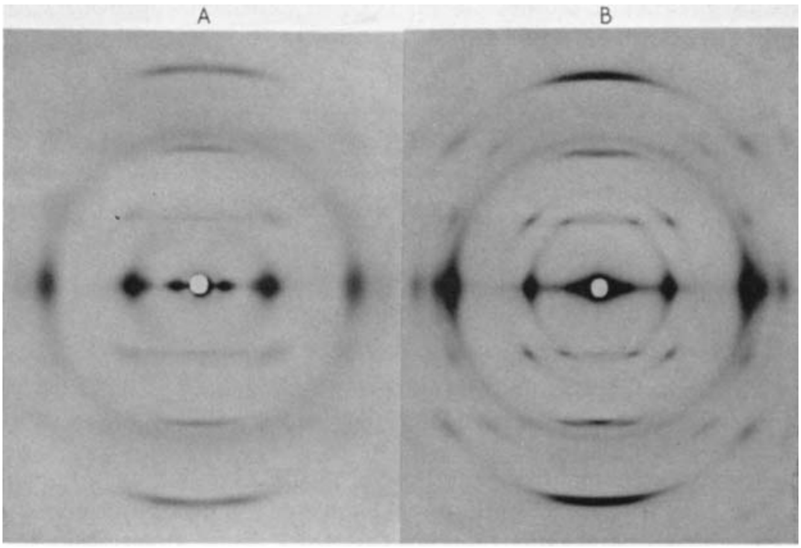

FIG. 12. X-ray photographs of well-orientated intersegmental cuticle of lobster. Vacuum camera. A. Before purification; B. After purification in dilute caustic alkali.

in the *c*-direction, the strong reflection at 33Å being the second order of this. On this basis we would account for the reflection about the 010 position as the pair of 011 reflections with $c \sim 66$Å. Indeed, the reflections near the meridian on the first layer line appear to be two slightly off meridional reflections and are in reasonable agreement with this 011 indexing. Along the first layer line there is, quite probably, a spread out series corresponding to various *01l* reflections, with perhaps odd orders being the strongest, e.g. 011, 013, 015. This cannot be confidently asserted but is in marked contrast to the definite reflections at 011 and 012 of the pure chitin lattice in B. The near-in reflections on the first

layer line in A suggest that protein is regularly arranged with reference to the polysaccharide chains, giving rise to definite lattice points which are the origin of the reflections we think of indexing as 011.

In Fig. 12A the meridional arc on the third layer line is definitely split in two and these appear to be the equivalent of the 031 reflections of pure chitin. But within the limits of accuracy of the present photographs it is not possible to define the indices for the chitin/protein complex. We would compare this with Fig. 1C, where the main contribution on the third layer line at the meridian should have the index of 012 where $c \sim 29$Å.

Many properties connected with the equatorial reflections can be more conveniently studied using line spectra. For example, Figs. 13A

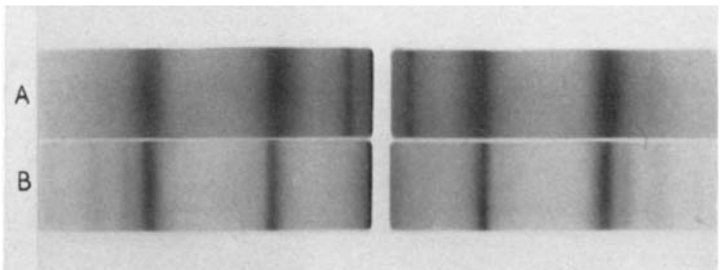

FIG. 13. Line spectra of blowfly larval cuticle with the X-ray beam parallel to the surface of the cuticle. Dried in air. A. Natural cuticle; B. After purification.

and B give the results for A, untreated air-dried cuticle of *Sarcophaga barbata*, and for B the same cuticle purified in boiling aqueous 5% potassium hydroxide and showing the well-defined sharp lines of the purified chitin spectrum. In A the chitin lines are broader and there is the very strong line near the centre at 33Å, which is absent in B where the protein is removed. This line at 33Å has suggested to some a lipid-type structure, perhaps even arising from the epicuticle. However, it is quite unaffected by scraping the external surface until all epicuticle would have been removed. And the most prolonged Soxhlet extraction with ether, alcohol, benzene or chloroform has no effect on the presence of this line. But the line is very easily caused to disappear by steaming the cuticle for a minute, by treating the cuticle in cold dilute acid or alkali, or by tanning the cuticle in cold aqueous benzoquinine or catechol. The spacing also disappears if the cuticle is repeatedly dried and rewetted several times. These various properties are those we should associate with a comparatively labile protein.

The special advantages for study of the larval cuticle in cyclorrhaphous flies is that in the puparium we can examine the same cuticle after it has been hardened by the tanning process. The cuticle of the puparium gives rise to essentially the same X-ray diffraction diagram as the larval cuticle; there is the same non-sharpness of equatorial and non-meridional reflections, strong well-defined reflection at 33Å on the equator and the sharp well-defined 011 type reflections. We are particularly interested in the behaviour of the major reflection at 33Å. Unlike the situation in the larval cuticle, here it is stable to boiling water, cold dilute acid and alkali or treatment with quinones; these things we can associate with the protein now being stabilized by tanning during the formation of the puparium. In cold water the spacing shows a change from 33Å to 45Å. This is a continuous change for intermediate positions are obtained with methanol, ethanol or with saturated ammonium sulphate solution. The spacing obtained after swelling in formic acid is very much greater, 60–70Å but is quite reversible to 33Å on washing with water. In the puparium the planes separated by 33Å lie approximately parallel to the surface. The percentage swelling, as measured by the change in the diffraction diagram, is very nearly the same as that measured with the microscope for thin sections of the whole cuticle thickness. A complete range of measurements, microscopic and in diffraction patterns, of the swelling caused by immersion in alcohol, water, dilute hydrochloric acid and formic acid would give an increasing series of values. If the two swelling measurements "optical" and "diffraction" agreed reasonably closely it would be certain that the layers of spacing 33Å were effectively continuous throughout the thickness of the cuticle.

The larval cuticle is often unsuitable for a lengthy series of experiments as it is less stable; for example, it shows irreversible changes in very dilute acid and alkali and on heating. The very stable cuticle of the puparium is, however, difficult to obtain in larger quantities free from unhardened endocuticle. A pupal case from which a fly has emerged has a thin colourless endocuticle which gives a diffraction diagram closely corresponding to that of purified chitin, presumably because moulting fluids have disrupted the chitin/protein complex in this layer. Nowhere else in a natural structure have we met an example of α-chitin in this condition, i.e. appearing like purified chitin. To obtain meaningful diffraction diagrams of puparium cuticle we have to scrape away thoroughly this layer. It is tedious and difficult because of the stiffness and comparative brittleness of the hardened wall of the puparium.

In Fig. 14 we show the effect of swelling in methanol on blowfly

larval cuticle and on well-scraped puparium cuticle. In both cases the inner two lines *c*, *c′* are distinctly moved towards the centre as in B, C and D, E. The other line *a* is scarcely visibly moved and this is to be expected in that it corresponds to the 101 reflection of Carlström (1957). The innermost line in A and F represents the 33Å spacing; it moves to a spacing of about 48Å in C and to about 42·5Å in D. These changes

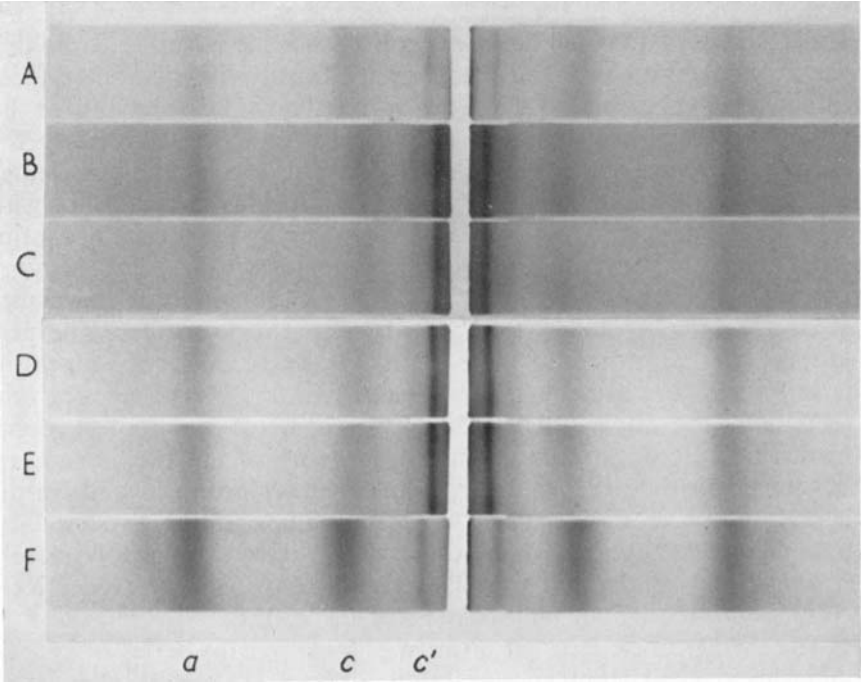

FIG. 14. Line spectra of larval cuticle A, B and C and of hardened puparium cuticle D, E and F. A. Air dried larval cuticle; B. Same in 95% methanol; C. Same in 92% methanol; F. Air dried puparium cuticle; E. Same in 92% methanol; D. Same in 80% methanol.

represent swellings of 30% and more. Equally noteworthy is the very pronounced shift in the broad line at the position of 002 in chitin, indicating an interplanar swelling of about 10%. According to our view the methanol is penetrating between the piles of chains A, B of Fig. 5, and also, but to a greater degree, at the protein/chitin interface, e.g. p-c of Fig. 16, giving a total swelling of about 30% for the puparium and somewhat more in the case of the larval cuticle treated in 92% methanol.

There are other details in the best diffraction diagrams of this type that are concerned with various orders of the reflection at 33Å. One phenomenon, which gives promise of leading to a better definition of the chitin/protein structure, is illustrated in Fig. 15. Under certain conditions of swelling the line, corresponding to the 002 reflection of pure chitin, becomes noticeably sharpened. This is seen at the level *c* in the middle spectrum B of Fig. 15. Figure 15A is dried blowfly larval cuticle, B the same specimen wet in saturated ammonium sulphate solution, and C is the same specimen wet in half-saturated ammonium

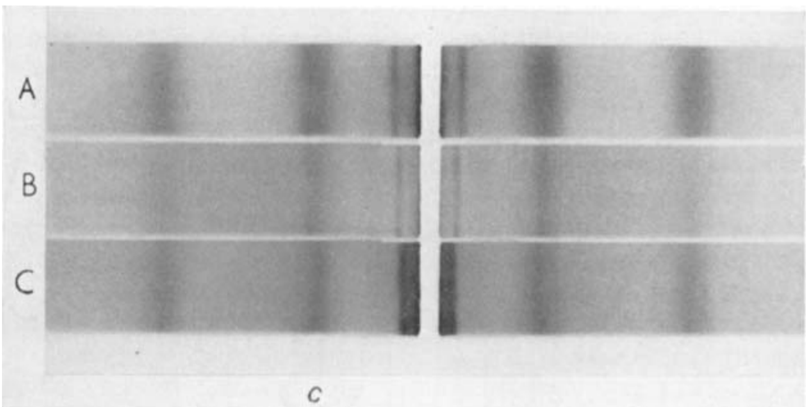

FIG. 15. Line spectra. Blowfly larval cuticle. A. Dried in air; B. Wet with saturated ammonium sulphate; C. Wet with half-saturated ammonium sulphate.

sulphate. In B the 002 line is sharp compared with the corresponding line in the dry specimen A or with that in more swollen specimen C. The diagrams Fig. 16A, B and C suggest how this phenomenon may occur. In A, sets of three chitin chains (c) are shown separated by a protein chain (p) and there is no continuous regular period p to c to c etc., the only regular period being p–p. But in a particular state of swelling we could have the condition in Fig. 16B, where there is a regular period p : c : c, etc. throughout the structure as well as the larger regular period p–p. In the more swollen condition, Fig. 16C, the only continuous regular period is the large scale one p–p. This type of argument may well apply in the case of Figs. 15A, B and C. Apart from any attempt at a specific interpretation, the fact that the sharpening occurs at a particular degree of hydration suggests the conditions under which the best and most meaningful diffraction data should be obtained.

The type of structure associated with these details has been found in the soft larval cuticle of all Diptera, Lepidoptera and Coleoptera so far examined. The main features of molecular organization are not altered by the process of tanning to form the blowfly puparium. Therefore one might expect to find this structure in many hardened cuticles, but it has not been found in any hard cuticle other than in blowfly puparia. We conclude that the X-ray diffraction diagram of Fig. 12A is associated only with cuticles that go through a stage like that in blowfly larva, or in typical caterpillar of Lepidoptera. The cuticle must be tough and function as a mobile deformable skin. We have not found the honey bee larval cuticle to have this particular structure; compared with the blowfly larva it is less tough and less capable of considerable extension without breaking.

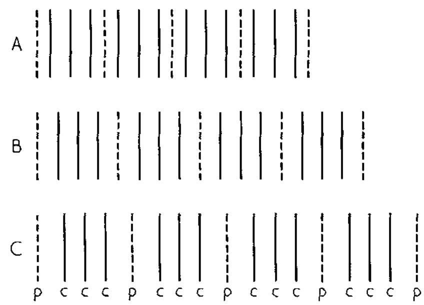

FIG. 16. Diagrams to suggest an interpretation of Fig. 7A, B and C. Protein chains, p; chitin chains, c.

B. INTERSEGMENTAL SOFT CUTICLE IN ADULT ARTHROPODS

In Fig. 12A we have used intersegmental cuticle from the ventral surface of lobster tail to illustrate the standard diffraction diagram obtained from soft larval cuticles. This is because both structures give the same type of X-ray diffraction diagram. They also have general physical properties in common; the intersegmental cuticle of the lobster is a tough deformable membrane, and when isolated it can be stretched considerably without breaking. These are the properties which are associated with the diffraction pattern we have considered in the previous section.

One might expect to find this same structure in intersegmental cuticle from adult insects. These are often rather small regions but they have been obtained in reasonable quantity from large adult mantids. Such isolated cuticle was tough and could be stretched without breakage

to an extension of 30–40%. The X-ray diffraction diagram was, in principle, indistinguishable from that given by stretched blowfly larval cuticle but the reflection on the equator was a little larger than 33Å being about 37Å. This enlarged spacing is commonly found in partly wet specimens or in dry specimens where sugars or ammonium sulphate or dyestuffs have been trapped in the lattice (Fraenkel and Rudall, 1947). It is evident that there is a recognizable molecular structure which is common to all these pliable yet tough cuticles which exist in conditions where strength and mobility is required, that is, in the cuticle of free living soft-bodied larvae and in the intersegmental membranes of adult arthropods.

C. HARDENED CUTICLE OF ADULT INSECTS

Hardened larval cuticle, as in the puparium of cyclorrhaphous flies, shows the same molecular organization as in the soft larval cuticle or the white pupa cuticle. So the process of hardening itself need not obscure characteristic features of the soft cuticle. But some artificial methods of hardening, such as using benzoquinone or catechol, destroy the equatorial reflections at 33Å in blowfly larval cuticles. It is just possible that in the hardening of some adult insect cuticles a special hardening agent alters the supposed regularity of structure occurring in the soft stage. We can only test such a possibility by examining adult cuticles before the hardening takes place.

We can make some generalizations about the X-ray diagram of hardened adult cuticles. It is not that of purified chitin in that the equatorial and non-meridional reflections are ill-defined as if the crystallites of chitin were very small in the lateral dimension. As far as can be judged, there are generally reflections near the 010 position though the very well-defined reflection on the equator at 33Å has not been detected.

In the following sections we describe two outstanding types of diffraction diagram from adult insects. These patterns are, of course, best defined in the most highly oriented pieces of cuticle, but the salient features can be detected in all hard parts which have been examined.

1. *Hymenopteran cuticle*

The ovipositor of the large wood-wasp *Sirex* provided the first comparatively high-quality diffraction diagrams of chitin/protein complexes. The long tubular structure gives the X-ray diagram shown on Fig. 17A and has the general features of a chitin/protein complex, namely, the reflections are generally ill defined and there is a fairly

well-defined reflection about the 010 position of the meridian. But the outstanding feature of Fig. 17A is the prominent series of reflections on or near the meridian; this series consists of nine distinct orders of a fundamental spacing of 31Å. The third, sixth and ninth orders coincide with the first, second and third layer lines of the chitin structure. Of the nine orders of the 31Å spacing the odd orders are stronger than the even orders; of course we cannot really specify the strength where there is coincidence with the strong reflections of the chitin structure.

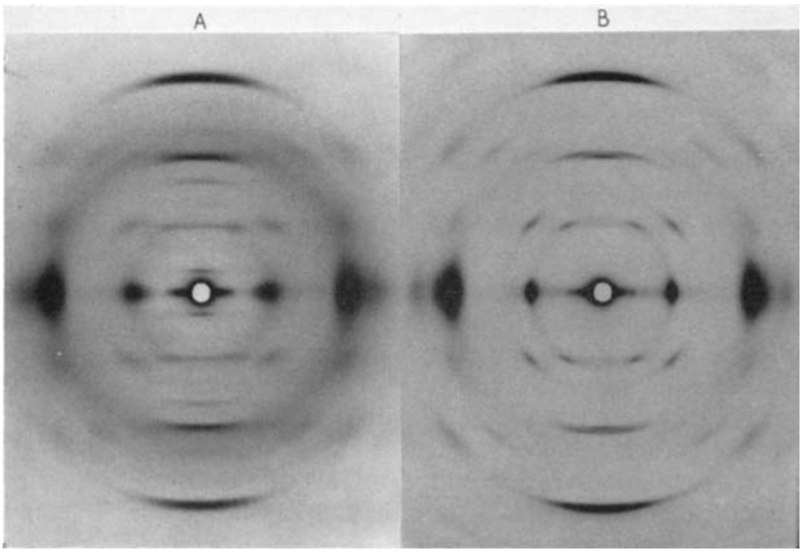

FIG. 17. X-ray photographs of *Sirex* ovipositor. Vacuum camera. A. The natural condition; B. After purification.

The most reasonable general conclusion is that protein fits exactly with the chitin lattice to give a repeating unit of the protein along the fibre axis of exactly six *N*-acetylglucosamine residues, i.e. 31Å. It is clear from Fig. 17A that there are various layer line reflections associated with the better defined arcs near the meridian.

It must be of interest to know if there is any larger regular structure in the fibre direction of the chitin itself. Purified chitin from the ovipositor is shown in Fig. 17B, and there is no suggestion of a larger period in the fibre axis direction other than the characteristic chitin period of 10·3Å, due to the chitobiose unit.

The type of structure shown in Fig. 17A is not peculiar to the

ovipositor of *Sirex*. It is also found in the cuticle of leg segments and in general body cuticle such as tergites. In fact, it is found in all Hymenopteran cuticle examined, e.g. in bumble bees, wasps and in the ichneumon, *Rhyssa*, which parasitises *Sirex*. It seems to be present equally in Apocrita and Symphyta, it being found in the leg segments of the gooseberry sawfly *Nematis ribesii*. The special structure with nine well-defined orders of a spacing of 31Å on the meridian thus appears in a whole insect order and could scarcely be missed in a reasonably well-produced X-ray diffraction photograph of any uniform piece of cuticle.

We had frequently examined cuticle of adult Diptera (*Tipula* leg segments, blowfly tergites) and cuticle from stick insect, cockroach and mantid. In none of these did we find special features in the diffraction pattern other than the usual poor definition of some reflections as is characteristic of chitin/protein complexes in general. But there were outstanding special features in the case of locust cuticle.

2. *Orthopteran cuticle*

Of the three main types of Orthopterous insect, grasshoppers, locusts and crickets, the locust and its cuticle are most frequently examined in the laboratory. In an earlier survey, samples of locust cuticle revealed special features in X-ray photographs and a reasonably good diagram from scraped leg segment cuticle is shown in Fig. 18. It shows the usual general characteristics of a chitin/protein complex, namely, broadness of non-meridional reflections and some intensity about the 010 position. The special feature is the large number of additional layer lines, and these indicate the same fundamental period of 31Å in the fibre axis direction. By comparing Fig. 18 with Fig. 17A it appears that in the locust cuticle it is the even orders of 31Å that tend to be intensified, while it is the odd orders in the Hymenopteran cuticle. Some structure having a fundamental axial period of 31Å is present in the two types of cuticle. The intensity distribution on the first two layer lines is obviously different; and the locust cuticle shows other meridional reflections than those agreeing with simple orders of 31Å. These may be other orders of a larger unit of which 31Å is a principal subunit, or they may belong to another separate structure. We meet a similar situation in the case of *Aphrodite* chaetae (p. 301).

In *Locusta migratoria* we are not able to detect a difference between the X-ray diffraction diagrams from gregarious and from solitary forms. We have not detected a difference between *Schistocerca gregarina* and *Locusta migratoria* as regards these prominent molecular features.

In other Orthopterous insects we have always detected the kind of diagram shown in Fig. 18, but the special series of meridional reflections is generally less prominent in the cuticle of crickets and long-horned grasshoppers than of locusts. There is a structure which is characteristic of Orthopteran cuticle but it is perhaps most clearly seen in Acrididae.

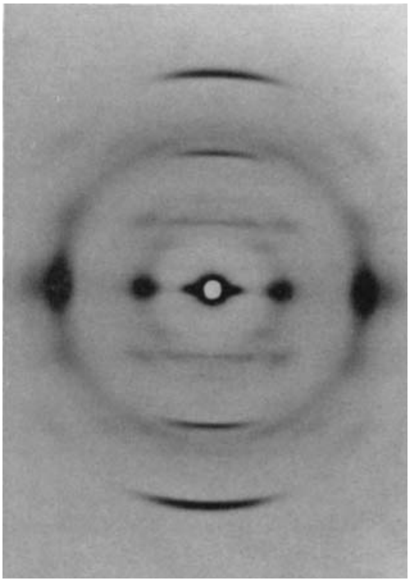

FIG. 18. X-ray photograph of leg segment cuticle from a locust. Vacuum camera.

D. OESOPHAGEAL CUTICLE OF THE SQUID, LOLIGO

There are probably other types of X-ray diffraction diagrams to be obtained from insect or arthropod cuticle than those we have described and illustrated in the previous sections A, B and C. But of the widely varied materials already examined the diffraction pattern of the chitinous cuticle lining the oesophagus of the Squid, *Loligo* is the most extraordinary we have met with in our studies of chitin/protein complexes. In it, the chitin diagram is modified or changed to a far greater degree than in our other examples.

The oesophageal cuticle of dead specimens of *Loligo* becomes detached from underlying tissues and can be drawn out as a tube and washed in water. In this fresh and moist condition it is easily extended to give the diffraction diagram of Fig. 19A; after removal of the

protein by boiling in 5% aqueous sodium hydroxide the diagram of purified α-chitin is obtained as in Fig. 19B. The pattern in Fig. 19A is quite unlike that of chitin; its main features could be those of many other polymers consisting of 1→4 β-linked pyranose units. The prominent meridional reflections of chitin on the second, third and fourth layer lines are there with the reflection at about 5·14Å on the second being distinctly more intense than that at about 3·3Å on the third layer line. These relative intensities are those characteristic of β-chitin. On the

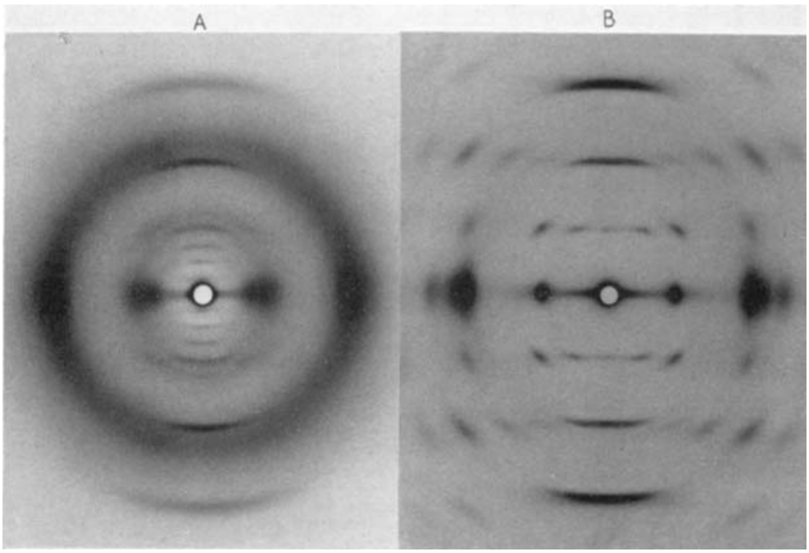

FIG. 19. X-ray photographs of *Loligo* oesophageal cuticle. Vacuum camera. A. The natural condition; B. After purification.

equator the main reflections are ~4·4Å, 10Å and 13Å. Non-axial reflections are nearly invisible, the best defined is that on the first layer line and approximately on the row line through the equatorial reflection at 13Å (Cf. Fig. 24). These things emphasize how very considerably different from chitin is the structure represented by Fig. 19A, yet removal of the protein leads immediately to the standard α-chitin structure as in Fig. 19B.

Figure 19A shows, in an extreme form, one of the characteristics of chitin/protein complexes, namely poor definition of non-meridional reflections. But here, too, the meridional reflections are unusual. We have noted that the second layer line reflection is stronger than that on the third layer line. But the most striking feature is the series of

meridional or near meridional reflections. These are well defined and sharp; they are various orders of a fundamental spacing of 41Å and orders 1, 2, 3 and 4 are very obvious, with some others being seen less clearly.

In the X-ray diagrams of Figs. 17 and 18 we dealt with complexes showing a fundamental axial spacing corresponding to the length of *six* pyranose residues. Here we are dealing with a unit of structure repeating in the direction of the fibre axis at 41Å, which is closely equal to the length of *eight* pyranose residues.

The *Loligo* oesophageal cuticle is a soft, colourless structure and therefore rather different from the hard sclerotized cuticles described in section C. Because of its position lining part of the alimentary canal it may well be very different in mechanical and physiological functions from the external cuticle of Arthropods. But it should be interesting to examine the fine structure of intact cuticle lining the gut of insects, crustaceans etc., to see how this compares with the oesophageal cuticle in the mollusc *Loligo*.

E. APHRODITE CHAETAE

Lotmar and Picken (1950) described a number of additional meridional reflections in the notopodal chaetae of *Aphrodite*. But there are many other more significant reflections on the meridian at smaller angles and these are shown in Fig. 20 (Cf. Fig. 24). The main meridional series gives a fundamental fibre axis period again of 31Å with the following orders being prominent: 1, 2, 3, 5, 7 and 9. There are a number of other special features in Fig. 20 which we might draw attention to in this brief account. The main *c*-axis reflection of the chitin near 10–11Å is split into two spots above and below the equator. And beyond these on the same row line are well-defined streaks. Of particular note are the well-defined spots on the first layer line of the chitin pattern proper, which indicate a principal period at right angles to the fibre axis of about 25Å.

On removing the protein, *Aphrodite* chaetae give a β-chitin diagram very like that in Fig. 1A. This we interpreted as a system where all the chains are parallel and with but a single chain running through the unit cell. The prominent spots on the first layer line mentioned in the last paragraph point to a regular geometrical arrangement of chitin chains and protein chains, that is, a single chitin/protein lattice. All the additional features in Fig. 20 can be attributed to the protein content and the diagram indicates the existence of one definable chitin/protein structure.

In Fig. 20 there are a few weaker reflections at larger angles (which might possibly be near meridional reflections) that are not orders of 31Å. Also there are various row line streaks which could indicate a larger unit than 31Å in the fibre axis direction. But it is clear that there is something particularly important about 31Å as a principal fibre axis period.

One is seeking the conditions in which the elaborate pattern in Fig. 20 can be obtained in its most perfect and complete form. One knows, for

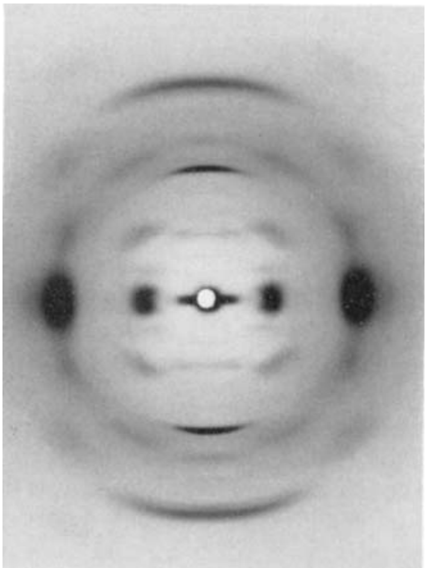

FIG. 20. X-ray photograph of natural *Aphrodite* chaetae. Vacuum camera.

example, that most of the detail is not found in the case of the uncoloured (untanned) chaetae or spicules which are found in *Aphrodite*; this might indicate that the properly stabilized condition of the protein is necessary for showing them. Normal well-tanned chaetae show a decreasing perfection of the diagram when treated in dilute hydrochloric acid, again indicating that the details of the complex structure are liable to be lost or to deteriorate.

V. OPTICAL STUDIES

Of the many details in microscope images of cuticle, the very marked discontinuities in structure due to the lamellae have raised questions

about the distribution of chitin and protein. Lamellae are stated by Richards (1951) to vary in thickness from 0·2 μ to 10μ, while Locke (1960) gives even thinner dimensions. In the lower range of thickness they are of the order of half the wavelength of light and should give rise to various interference effects.

An attempt to define the relative position of protein and chitin, in units of structure like the lamellae, has been made by Pfaff (1952) and he gives schematic diagrams for cockroach and cockchaffer cuticle. One can see how these conclusions have been reached, but in view of the picture emerging from modern electron microscope studies, one concludes that Pfaff's diagrams are too simplified and rather misleading. An important part of Pfaff's argument concerns the sharpening of the X-ray diagram when endocuticle is treated with pepsin; this change is attributed to the removal of protein by the proteolytic enzyme. We had discovered this effect in the case of blowfly cuticle, namely, if one removes or alters the protein the crystalline pattern of pure chitin appears in place of a more diffuse X-ray diagram. A sharpening of the diagram can also be effected by steaming, soaking in cold dilute acid, etc., so in Pfaff's treatment of cockroach endocuticle we do not know if the changes seen by X-rays are due to the low pH or to the pepsin digestion.

Among microscopic studies we should recall again the very important paper by Dennell (1946) on the growth of the third instar larval cuticle in blowflies. This indicated that new lamellae can be laid down in middle layers of the cuticle at considerable distances from the epidermal cells and through an inner endocuticle devoid of pore canals. The further resolution of these problems is of interest in connection with chitin/protein complexes. What kind of fluid molecule is this that diffuses through the lower endocuticle? It would seem that it should be extractable during this "migratory", non-polymerized phase, and capable of definition as a macromolecule. Indeed, most of the "Schmidt layer" between epidermis and endocuticle seems to be in an incompletely aggregated state and could probably be brought into solution without "denaturation" of its constituent macromolecules. (See, for example, Locke, 1960.)

A. POLARIZATION OPTICS

Lotmar and Picken (1950) have made some very satisfactory measurements of birefringence (path difference) on *Aphrodite* chaetae. According to our view this material contains a well-defined chitin/protein complex and it gives the very detailed diffraction diagram shown in Fig. 20.

Lotmar and Picken used their results as an argument that protein in the cuticle was oriented and has positive intrinsic birefringence. They proceeded by measuring the "path difference" for intact untreated bristles and for the same bristles after extraction with potash, which were then effectively pure β-chitin. The untreated bristles were almost impervious to the range of imbibing fluids used, while the purified chitin was readily penetrated by the various fluids. We are drawing attention to their results, Fig. 21, as they form a fine standard of reference by which to judge what might be done using polarization methods to

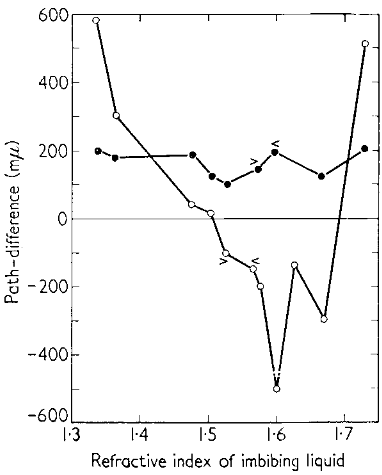

FIG. 21. Birefringence of *Aphrodite* chaetae before and after purification (Picken and Lotmar). ●—● The natural chitin/protein structures; ○—○ The chitin only, after purification.

throw further light on chitin/protein complexes. Had the separate points been obtained from a number of bristles instead of from single bristles, the curves would have a more normal smoothed-out appearance. Perhaps one might stress that the combined use of X-ray diffraction studies, polarization optics and the measurement of infra-red absorption dichroism should be the best approach to achieving really detailed interpretations.

Richards and Pipa (1958) have presented a number of specific models to account for some features of chitin/protein complexes. Their main arguments are based on birefringence measurements:

Purified chitin	0·00084
Intact cuticle	0·00070
Isolated cuticle protein (arthropodin)	0·00013

All the above measurements were made with specimens immersed in ethanol. The conclusion reached was that the only way to explain the birefringence of the cuticle is by subtracting the value for "arthropodin" from that for the chitin; that is, protein chains should be at right angles to chitin chains.

It would be very attractive to be able to establish such a fact but the conclusion is not justified. By consulting Lotmar and Picken's curves, Fig. 21, we see that with ethanol (R.I. 1·36) chitin is indeed more birefringent than the chitin/protein complex. But if an imbibing fluid of R.I. 1·45 had been used instead of ethanol, exactly the opposite result would have been obtained. We might summarize Fig. 21 by saying that the chitin/protein complex has just one value for birefringence, while the chitin itself has many different values depending on the imbibing fluid used.

VI. Electron Microscopy

Since the first successful studies of cockroach cuticle by Richards and Anderson (1942) the electron microscope has been very greatly improved as regards usable resolving power and the techniques of sectioning, staining and indeed many other arts of preparation have reached a high standard. The lamellae in the cockroach cuticle were about 0·2 μ thick and appeared as alternating layers of denser and less dense cuticular material. Naturally, these layers prompted questions such as the possibility of chitin-rich and perhaps chitin-free layers. Also relevant to our present considerations was the opinion that discrete fibrils, so obvious in electron microscope studies of cellulose membranes, were not present in unaltered cuticular material and this was considered as evidence for a chitin/protein continuum (Richards and Pipa, 1958).

The recent study by Locke (1961) is concerned with many aspects of cuticle structure; some very clear pictures are obtained of the lamellae in a number of cuticles and Locke describes abundant fine fibrils making up the substance of each lamella. In *Calpodes* larval cuticle "the lamellae are variably spaced from 0·1 μ to 1 μ apart". They are composed of "microfibres not more than 25Å in diameter" (Locke, 1960). While we might doubt the exactness of this figure, the order of size is that we have found in other caterpillar cuticles by X-ray means, namely 33Å for the separation of layers lying parallel to the surface of dried cuticle. Also there are pattern repeats of 45Å from planes approximately perpendicular to the surface in similar types of cuticle, e.g. from "white pupa" or puparium of blowflies (Fraenkel and Rudall, 1947). The figure of about 25Å is also of interest in connection with the possible

existence of a pile of "three segment" chains as in Fig. 3, the width of which should be somewhat under 30Å; or sometimes we might be dealing with "two segment" chains as in Fig. 4.

Smallness of fibrils is just what we require in order to explain the lack of sharpness in the X-ray diagrams at right angles to the fibre axis. The X-ray and electron microscope results are beginning to make useful contact, and in appropriate material we may perhaps obtain images of the fine structure along the fibre axis repeating at 31Å.

Richards has always maintained that there are no fibrils of chitin in the natural cuticle, and he gives a picture of what he means by fibrils (Richards and Pipa, 1958) where these have been produced by altering the cuticle chemically. One asks if the fibres described by Locke (1960, 1961) are not really profiles of "sheets" or layers cut transversely. A view of sets of sheets is given in Fig. 22 and in these we think of the

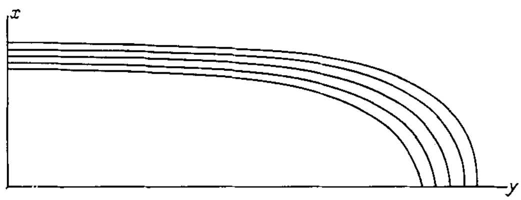

FIG. 22. Diagram of one form of "fanned out" layers. x-Axis perpendicular to the supposed surface of the cuticle; y-axis parallel to this surface.

chitin chains as being oriented perpendicularly to the surface of the paper with the a-axis in the plane of the layers and the c-axis perpendicular to these layers. We suggest this in order to correlate some X-ray diffraction results with the curious form of the profiles in electron micrographs, which "fan out at angles up to 90°" according to the description by Locke (1960).

X-ray diffraction photographs of dry puparium cuticle, A, or of dry white pupa cuticle, B, taken with the beam parallel to the transverse axis of the puparium and parallel to the surface of the cuticle, appear as in Fig. 23. We are looking down the length of the chitin chains and spacings of layers parallel to the cuticle surface are recorded along the x-axis and spacing of layers perpendicular to the surface are recorded along the y-axis. The unusual feature in the photographs A and B is the well-defined, oval form of the diffraction "ring" near the centre as if spacings were a minimum between layers parallel to the surface, a maximum between layers perpendicular to the surface and with a

clearly shown progressive increase of spacing as layers changed from being parallel, to being perpendicular to the surface.

This description fits the form of the layers in Fig. 22, which we are supposing are represented by the profiles in Locke's electron micrographs. The change from x to y in Fig. 22 takes place over a distance of 1 to several μ, which is small compared with the size of the X-ray beam, so that all layer slopes would contribute to the photographs. The "fanning out" of the layers is sufficiently gradual to give reasonably parallel equidistant layers, the separation increasing with the angle the layers make with the surface. We have long considered the possibility of this and equivalent superposed layer forms as an explanation of the diffraction pattern in Fig. 23A and B. We should describe a further

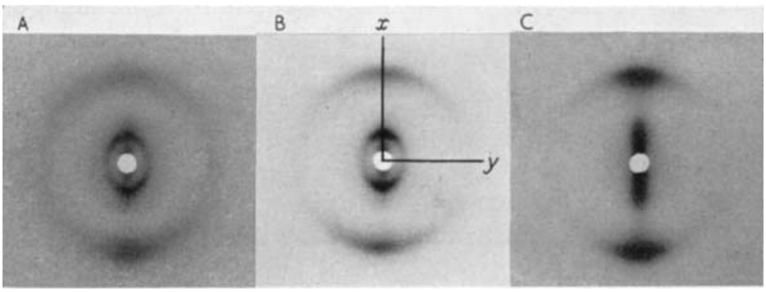

FIG. 23. X-ray photographs of blowfly puparium cuticle with the beam parallel to the transverse axis of the puparium and parallel to the surface. Cuticle dried in air. A. From hard brown puparium; B. From white pupa; C. White pupa cuticle pressed between glass while moist and then dried. x-Axis perpendicular to surface; y-axis parallel to surface.

property of the cuticle layers which are separated by 33Å. Compression has several interesting effects, one of which is illustrated on Fig. 23C. Here, fresh, moist white pupa cuticle was compressed between glass plates and dried. The "oval" around the central spot in Fig. 23B has now been replaced by a broad streak. The structure has somehow collapsed as a result of compressing. If we imagine that the compression brings the fanned out layers of Fig. 22 all more closely parallel to the surface, then we would have a variety of differently spaced layers parallel to the surface and the observed diffraction should be a streak oriented as in Fig. 23C.

There are a number of difficulties in relating the "fanned out" layers of the electron microscope image with the "oval" diffraction contours of Fig. 23. However, it may serve a useful purpose to place the two kinds of information side by side.

VII. Summary

The present article is concerned with biophysical aspects of the insect cuticle proper, neglecting the epicuticle. It considers the principal known facts about chitin and the cuticular proteins. It also gives a description of some new features of the chitin/protein complex as seen by X-ray analysis.

Three distinct forms of chitin, α, β and γ are described, these being distinguished crystallographically. Respectively in β-, α- and γ-chitins the long chains are grouped in sets of one, two and three. The explanation suggested for the γ-form is that a single chain is folded upon itself to give a unit consisting of three parallel segments (Fig. 3). Once having granted the existence of such folded polysaccharide chains, then it is possible that chitins which seem to be of α-chitin structure by X-rays, may actually consist of chains folded in various ways, namely, with two, three, four or more segments per chain. Perhaps of most interest to us is the three segment folded form of Fig. 3 with appropriate orientation of the triads to give the α-structure.

The β-form, with chains having the same direction, could be explained by supposing isotactic growth from a surface (e.g. see Natta, 1957). In this, new sugar residues would be added at a protein surface between this and the basal end of a lengthening polysaccharide chain growing out from the surface. In fact, the *Aphrodite* bristle would seem to fit this model as it grows out progressively from the base of the bristle follicle. But in other β-chitins, i.e. *Loligo* pen and Pogonophore tubes, we cannot directly visualize one end of the chain as being distant from, and the other end as being attached to a surface at which monomer units were being added. When parallel, single-direction chains have achieved a considerable length we cannot conceive of some completely changing their direction in order to give the alternate up and down chains of the α-chitin structure. Established chains of some length could, by folding upon themselves, give rise to γ-chitin as in Fig. 3 and this may be brought about by the chain being fixed at its two ends to protein. Both β- and γ-chitin are looked on as arising from a system of parallel as distinct from antiparallel chains. The simplest explanation for α-chitin would be that of random direction of chains in a "pool" out of which they become associated in the most stable form, i.e. the antiparallel α-structure. It seems that the α-structure would always be found when chitin chains are precipitated from solution. In natural structures, chitin chains are held by covalent links to protein and this circumstance may be the cause of chains having a common direction as in β-chitin, or being folded or grouped in threes as in γ-chitin.

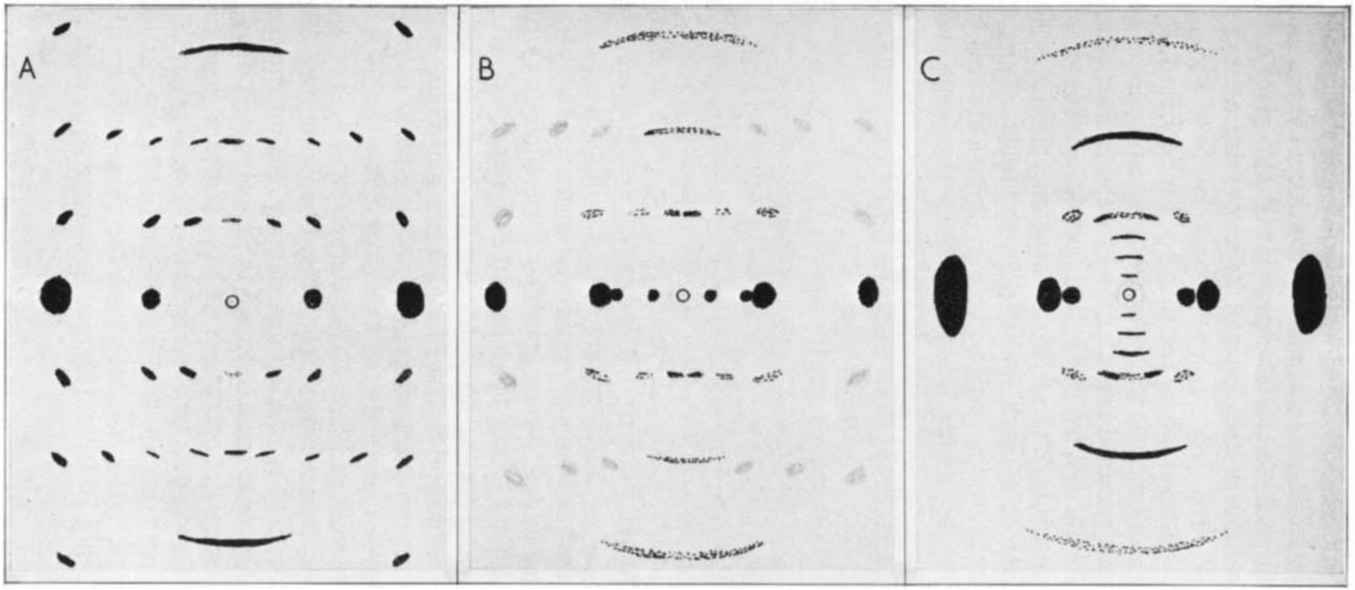

FIG. 24. Comparison of some main features in X-ray diffraction diagrams of chitin/protein complexes. Specimen to (flat) film distance only approximately the same. A. Purified α-chitin from *Loligo* oesophagus cuticle; B. Stretched intersegmental cuticle of lobster; C. Natural *Loligo* oesophagus cuticle, stretched.

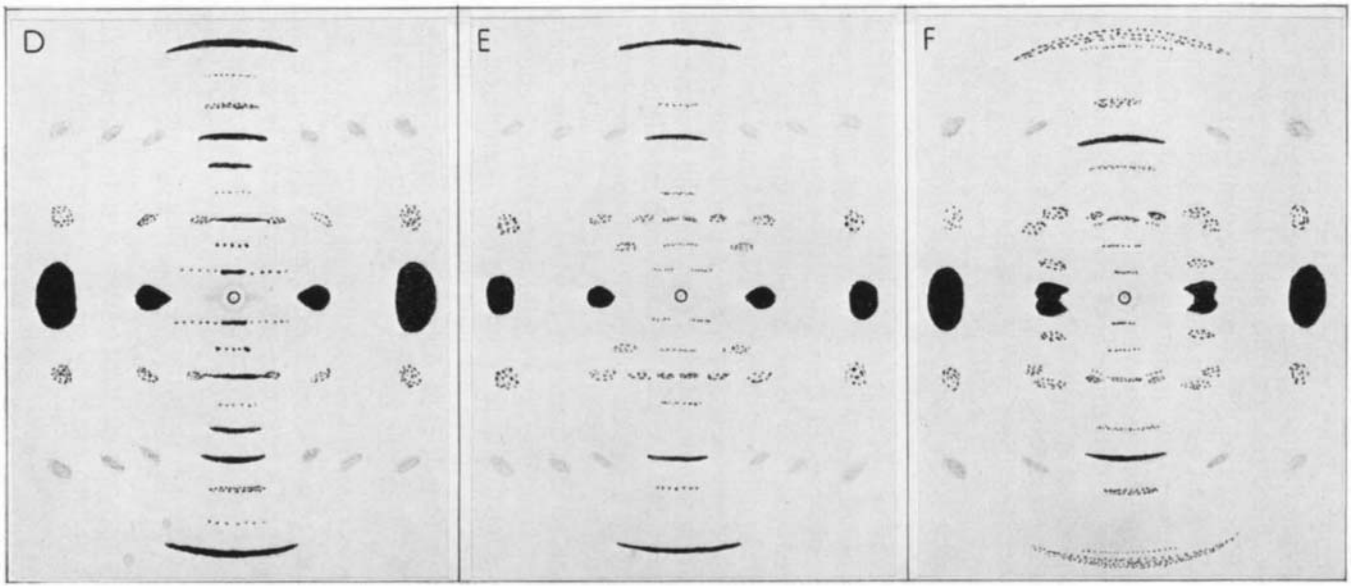

Fig. 24 (*cont.*). D. *Sirex* ovipositor; E. Locust leg-segment cuticle; F. *Aphrodite* chaetae.

We have given reasons for considering that chitin may possibly depart significantly from an idealized poly-*N*-acetylglucosamine structure. Analysis of elements had suggested that about one residue in six is deacetylated and that there is possibly about 5% bound water in thoroughly dried α-chitin. This is consistent with infra-red evidence for the presence of bound water in chitins. The bound water, as it were, replaces acetyl groups to maintain a density and crystallographic parameters appropriate to poly-*N*-acetylglucosamine. We have explained that this new view of the structure of chitin is not wholly satisfactory. Yet it has properties that would be consistent with the X-ray results for chitin/protein complexes where, in three instances, Hymenopteran cuticle, Orthopteran cuticle and *Aphrodite* chaetae, we have found a major period along the fibre axis of 31Å. This suggests that every sixth residue along the chitin chain could be different, i.e. glucosamine instead of *N*-acetylglucosamine.

In Fig. 24 a summary of the special features of chitin/protein complexes is given in diagrammatic form. The main diffraction spots of various X-ray photographs presented in this article were "inked in" and the silver image reduced. For comparison purified α-chitin from *Loligo* oesophagus (cf. Fig. 1B) is shown in Fig. 24A and the other diagrams of chitin/protein complexes, B–F, indicate how some of the reflections are altered, but more particularly they show the presence of additional reflections. Figure 24B is typical of many soft mobile larval cuticles and adult intersegmental membranes. It is characterized by the strong equatorial reflections of spacing ~33Å (cf. Figs. 12A, 13, 14 and 15). The other characteristic feature is moderately strong, near-meridional arcs on the first layer line corresponding to *011* reflections. No longer spacings in the direction of the fibre axis have been detected in this type of cuticle. Figure 24C shows the most considerably modified chitin type pattern we have found. It is dominated by axial periods which are orders of 41Å, this being equivalent to eight 1–4 β-linked pyranose residues.

Figures 24D, E and F all show a fundamental axial period of 31Å equivalent to six pyranose residues. Figures 24D and E are typical of Hymenopteran and Orthopteran cuticle respectively, and in both cases the chitin is in the α-form. Figure 24F is of *Aphrodite* chaetae and in this case the chitin is in the β-form: it is obvious that there are considerable complications of structure in the natural intact material compared with the purified β-chitin (cf. Fig. 1A).

These diagrams are consistent with a structure in which protein fits exactly on the pattern made by small groups of chitin chains. Where

the pattern of the chitin is extensive, as along the fibre axis, the enhancement of reflections due to regularly arranged protein is obvious. Only sometimes is regular arrangement particularly prominent at right angles to the fibre axis, e.g. Fig. 24B. Nevertheless there seems to be the possibility of more accurately defining the regular association of chitin and protein by finding those conditions of hydration which yield the most perfect diffraction patterns.

The problems of the association of protein with chitin are much more than the existence of a covalent link between them, important as this is. The details of the chitin/protein complex of soft mobile cuticles are very readily destroyed by weak acid or by steaming, while these same features have become very stable as a result of the natural tanning process. It is more the particular stability of the protein that maintains a stable chitin/protein association rather than the stability of one link between chitin and protein. These facts tell us that a number of weaker linkages are concerned in the natural association of chitin and protein.

We are particularly interested in the periodicity of 31Å parallel to the chitin chains as it may turn out to be a "master structure" here and in other fibrous structures. This and other features indicate how complicated are the details of structure in just one part of the whole cuticle. Perhaps the chitin/protein association is as complex as that of say actin and myosin, without there being particular reason to think of the cuticle proper as having any dramatic properties. But it is fabricated by molecular processes and, as we would expect, it is "accurately designed to the last detail".

Acknowledgment

Acknowledgment is made of support by the U.S. Public Health Service Grant, GM-07399.

References

Brunet, P. (1952). *Science* **116,** 126. Periodic acid-Schiff reaction in the insect cuticle.

Bunn, C. W. and Garner, E. V. (1947). *Proc. roy. Soc.* A **189,** 39–68. The crystal structure of two polyamides ("nylons").

Carlström, D. (1957). *J. Biophys. Biochem. Cytol.* **3,** 669–83. The crystal structure of α-chitin.

Carlström, D. (1962). *Biochim. Biophys. Acta* **59,** 361–4. The polysaccharide chain of chitin.

Clark, G. L. and Smith, A. F. (1936). *J. phys. Chem.* **40,** 863–79. X-ray diffraction studies of chitin, chitosan and derivatives.

Darmon, S. E. and Rudall, K. M. (1950). *Disc. Faraday Soc.* **9,** 251–60. Infra-red and X-ray studies of chitin.

Day, C. D. (1946). *Northw. Nat.* **21,** Suppl. 1–32. British Tachinid flies (Tachinidae (Larvaevoridae)).

Dennell, R. (1946). *Proc. roy. Soc.* B **133,** 348–73. A study of an insect cuticle.

Dweltz, N. E. (1960). *Biochim. Biophys. Acta* **44,** 416–35. The structure of chitin.

Dweltz, N. E. (1961). *Biochim. Biophys. Acta* **51,** 283–94. The structure of β-chitin.

Engström, A. and Finnean, J. B. (1958). "Biological Ultrastructure." Academic Press, New York.

Foster, A. B. and Hackman, R. H. (1957). *Nature, Lond.* **180,** 40–1. Application of ethylenediaminetetra-acetic acid in the isolation of crustacean chitin.

Fraenkel, G. and Rudall, K. M. (1940). *Proc. roy. Soc.* B **129,** 1–35. A study of the physical and chemical properties of the insect cuticle.

Fraenkel, G. and Rudall, K. M. (1947). *Proc. roy. Soc.* B **134,** 111–43. The structure of insect cuticles.

Giles, C. H., Hassan, A. S. A., Laidlaw, M. and Subramanian, R. V. R. (1958). *J. Soc. Dy. Col.* **74,** 647–54. Adsorption at organic surfaces. III. Some observations on the constitution of chitin and on its adsorption of inorganic and organic acids from aqueous solution.

Gottschalk, A. (1960). *Nature, Lond.* **186,** 949–51. Correlation between composition, structure, shape and function of salivary mucoprotein.

Gottschalk, A., Murphy, W. H. and Graham, E. R. B. (1962). *Nature, Lond.* **194,** 1051–3. Carbohydrate-peptide linkages in glycoproteins and methods for their elucidation.

Hackman, R. H. (1953). *Biochem. J.* **54,** 362–67. Chemistry of insect cuticle. I. The water-soluble proteins.

Hackman, R. H. (1959). *Proc. 4th Int. Congr. Biochem.* **12** (ed. L. Levenbook), 48–62. Biochemistry of the insect cuticle.

Hackman, R. H. (1960). Studies on chitin. IV. The occurrence of complexes in which chitin and protein are covalently linked.

Hackman, R. H. and Goldberg, M. (1958). *J. Insect Physiol.* **2,** 221–31. Proteins of the larval cuticle of *Agrianome spinicollis* (Coleoptera).

Heyn, A. N. J. (1936). *Protoplasma* **25,** 372–96. Further investigations on the mechanism of cell elongation and the properties of the cell wall. IV. Investigations on the molecular structure of chitin cell wall of sporangiophores of Phycomyces and its probable bearing on the phenomenon of spiral growth.

Keller, A. and O'Connor, A. (1958). *Disc. Faraday Soc.* **25,** 114–21. Studies of single crystals and their associations in polymers.

Liang, C. Y. and Marchessault, R. H. (1959). *J. Polym. Sci.* **37,** 335–395. Infra-red spectra of crystalline polysaccharides. I. Hydrogen bonds in native celluloses.

Locke, M. (1960). *Quart. J. micr. Sci.* **101,** 333–8. The cuticle and wax secretion in *Calpodes ethlius* (Lepidoptera, Hesperidae).

Locke, M. (1961). *J. Biophys. Biochem. Cytol.* **10,** 589–618. Pore canals and related structures in insect cuticle.

Lotmar, W. and Picken, L. E. R. (1950). *Experientia* **6,** 58–9. A new crystallographic modification of chitin and its distribution.

Manley, R. St. John (1961). *Nature, Lond.* **189,** 390–1. Crystals of cellulose.

Marchessault, R. H. and Liang, C. Y. (1960). *J. Polym. Sci.* **43,** 71–84. Infra-red spectra of crystalline polysaccharides. III. Mercerized cellulose.

Marchessault, R. H., Morehead, F. F. and Walter, N. M. (1959). *Nature, Lond.* **184,** 632–3. Liquid crystal systems from fibrillar polysaccharides.

Marchessault, R. H., Pearson, F. G. and Liang, C. Y. (1960). *Biochim. Biophys. Acta* **45,** 499–507. Infra-red spectra of crystalline polysaccharides. VI. Effect of orientation on the tilting spectra of chitin films.

Marchessault, R. H., Morehead, F. F., Walter, N. M., Glaudemans, C. P. J. and Timell, T. E. (1961). *J. Polym. Sci.* **51,** S66–8. Morphology of xylan single crystals.

Meyer, K. H. and Pankow, G. W. (1935). *Helv. chim. acta* **18,** 589–98. Sur la constitution et la structure de la chitine.

Moorefield, H. H. (1953). Studies on some integumental proteins of insects. Ph.D. Thesis, University of Illinois.

Natta, G. (1957). *Sci. Amer.* **197,** 98–104. How giant molecules are made.

Parker, K. D. and Rudall, K. M. (1957). *Nature, Lond.* **179,** 905–6. Structure of the silk of *Chrysopa* egg-stalks.

Pearson, F. G., Marchessault, R. H. and Liang, C. Y. (1960). *J. Polym. Sci.* **43,** 101–116. Infra-red spectra of crystalline polysaccharides. V. Chitin.

Pfaff, W. (1952). *Höfchenbr. Wiss.* **5,** 93–160. Investigation of the construction of the cuticles of insects and of the mechanism of penetration by the contact insecticide E605.

Richards, A. G. (1951). "The Integument of Arthropods". Univ. Minnesota Press.

Richards, A. G. (1952). *Science, Lond.* **115,** 206–8. Studies on orthropod cuticle. VII. Patent and masked carbohydrate in the epicuticle of insects.

Richards, A. G. and Anderson, T. F. (1942). *J. Morph.* **71,** 135–84. Electron microscope studies of insect cuticle, with a discussion of the application of electron optics to this problem.

Richards, A. G. and Pipa, R. L. (1958). *Smithson. misc. Coll.* **137,** 247–62. Studies on the molecular organization of insect cuticle.

Rudall, K. M. (1955). *Symp. Soc. exp. Biol.* **9,** 49–71. The distribution of collagen and chitin.

Rudall, K. M. (1962a). "The Scientific Basis of Medicine Annual Reviews", 203. Athlone Press, London.

Rudall, K. M. (1962b). *In* "Comparative Biochemistry", **4** (eds. Florkin and Mason). Academic Press, New York. Silk and Other Cocoon Proteins.

Runham, N. W. (1961a). *J. Histochem. Cytochem.* **9,** 87–92. Investigations into the histochemistry of chitin.

Runham, N. W. (1961b). *Quart. J. micr. Sci.* **102,** 371–80. The histochemistry of the radula of *Patella vulgata.*

Tracey, M. V. (1955a). *In* "Modern Methods of Plant Analysis" **2,** 264 (eds. Paech and Tracey). Springer, Berlin. "Chitin".

Tracey, M. V. (1955b). *Biochem. J.* **61,** 579–86. Chitinase in some Basidiomycetes.

Trim, A. R. (1941). *Biochem. J.* **35,** 1088–98. Studies of the insect cuticle. I. Some general observations on certain arthropod cuticles with special reference to the characterization of the proteins.

Waterhouse, D. F., Hackman, R. H. and McKellar, J. W. (1961). *J. Insect Physiol.* **6,** 96–112. An investigation of chitinase activity in cockroach and termite extracts.

Wigglesworth, V. B. (1956). *Quart. J. micr. Sci.* **97,** 89–98. The haemocytes and connective tissue formation in an insect, *Rhodnius prolixus* (Hemiptera).

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Osmotic and Ionic Regulation in Insects

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I. Introduction

The problem of the maintenance of salt and water balance in insects is of especial interest to comparative physiologists in view of the wide variety of different environments in which these animals are found. The majority of adult insects are terrestrial and their habitats range from moist to excessively dry conditions. The larvae of many species are, however, aquatic; most of them inhabit fresh water, but a few (often closely related to the freshwater forms) are adapted to life in saline waters. Some of the adult insects are also aquatic or amphibious.

The chief difficulty in the experimental study of osmotic and ionic

regulation in insects has been their small size, but in recent years this has been largely overcome by the development of ultra-micro analytical techniques. The use of these methods, together with the employment of radioactive tracers, has contributed very greatly to recent advances in this field.

Already excellent accounts of the subject of this review have been given in the text-books by Krogh (1939), Wigglesworth (1953) and Roeder (1953) and no useful purpose is served by repeating them. This review is therefore limited to a detailed consideration of the more recent work, using older studies as a basis for the understanding of later developments. Also the subject of water relations of insects has been reviewed (Edney, 1957) and, more recently, Beament (1961a) has discussed the nature of the insect cuticle in relation to water movement. This subject will, therefore, only be briefly mentioned. Finally, the organic constituents of insect haemolymph will be considered only from the aspect of their rôle in osmotic regulation.

The total concentration of solutes in the haemolymph of insects is not dissimilar from that found in a wide variety of other terrestrial and freshwater animals. The haemolymph freezing point depression lies between 0·4 and 1·3°C (see Buck, 1953). These limits are equivalent to concentrations of sodium chloride of about 120 and 376 mM/l respectively, or to solutions between about one-third and two-thirds the strength of sea water.

The majority of insects which have been studied maintain their haemolymph concentration within these limits irrespective of their habitat.

The problems encountered in the maintenance of a relatively constant internal medium are well known and clearly related to the nature of the external environment. Thus terrestrial animals must acquire water and salts from their environment to offset losses by evaporation through the body surface and elimination via the excretory system. Freshwater animals also obtain salts from the environment but must be able to eliminate water which enters the blood osmotically. In salt-water insects the situation is reversed—the maintenance of the normal haemolymph composition requires the uptake of water and the elimination of salt. Adaptation to life in any of the three major types of environment clearly presents its own special problems—problems which are closely bound up with the process of excretion.

The experimental work on osmoregulation in insects may be conveniently considered under two headings. Firstly, studies primarily aimed at assessing the ability of the animal to regulate its internal

composition. This approach involves the measurement of the composition of the haemolymph under different environmental conditions and has been initiated by the studies of Wigglesworth (1938) on regulation in freshwater mosquito larvae, and by Beadle (1939) on similar problems in a salt-water species. Secondly, studies designed to elucidate the mechanisms by which regulation is achieved. This includes investigations on the rôle of those regions of the body, such as the integument, the gut and the excretory system, through which uptake and loss of water and solutes takes place. Here, mention may be made of the work of Koch (1938), who first described the active uptake of ions by the anal papillae of mosquito larvae, and of the masterly studies by Wigglesworth (1931a, b, c; 1932), by Ramsay (1950-8) and by Phillips (1961), from which the main features of the physiology of the insect excretory system have emerged. In those organs concerned with the maintenance and regulation of the normal haemolymph composition, the movement of water and solutes takes place either passively—down concentration or osmotic gradients—or through active transport systems specifically adapted for the uphill transport of particular substances. The maintenance of an internal environment which is not in thermodynamic equilibrium with its surroundings depends on the presence of such transport systems, and the degree of regulation which can be achieved is related to the extent to which these systems are under physiological control.

II. The Characterization of Active Transport Systems

In insects, active transport systems are involved in the uptake of ions from dilute solutions by some aquatic forms and in the formation of the excretory fluid. Some preliminary consideration must therefore be given concerning how they may be characterized. Definitions of active transport have been put forward by a number of authors (see, for example, Rosenberg, 1954; Andersen and Ussing, 1960). Unfortunately, as our knowledge of the passive movement of substances across membranes increases, definitions of active transport become progressively more negative in character. Thus if we are to say (following Andersen and Ussing) that a substance can be regarded as actively transported only if the transfer of the substance cannot be accounted for by the action of purely physical forces, we find that, for any given situation, it is difficult to apply this definition unless we have precise knowledge of all the physical forces involved.

It would appear that the best we can do at present is to attempt to establish some criteria for the characterization of active transport which

take into account known forces and which can be applied to the available data.

A. ACTIVE TRANSPORT OF IONS

The direction of the passive movement of an ion is determined by forces which arise from the presence of concentration and electrical potential gradients, providing there is no interaction between the ions and other substances (such as water) which are crossing the membrane at the same time. If interaction takes place then the frictional forces acting between the moving substances (drag effects) are also involved (Ussing, 1952).

In the absence of frictional drag, the driving force for passive movement of an ion (its electro-chemical potential gradient, $\Delta\mu$) is given by the relation (Ussing, 1949):

$$\Delta\mu = RT\ln(a_1/a_2) + zFE \quad (1)$$

where a_1 and a_2 are the chemical activities of the ion in the solutions (1 and 2) on either side of the membrane, E is the potential difference across it (its sign being negative if it opposes the movement of the ion), z is the valency of the ion and R, T and F have their usual meanings. $\Delta\mu$ can be calculated from measurements of the potential difference and of the concentration ratio (c_1/c_2), assuming this to be approximately equal to a_1/a_2. If $\Delta\mu$ is positive then the expected passive movement is from solution 1 to solution 2.

If there is a net movement of water, solvent drag may be important (Ussing, 1952; Koefoed-Johnsen and Ussing, 1953; Andersen and Ussing, 1960). The quantitative treatment devised by these authors cannot be applied in most situations as the required membrane parameters are not known. Instead, the importance of this factor can usually be assessed by qualitative tests. Experimentally, the net water flow can be abolished by adjusting the osmotic pressure of one of the solutions (e.g., by the addition of sucrose) and the effect on the movement of the ion observed. A second test involves a simple calculation: if the ion movement results from solvent drag then the concentration of the ion (C_m) in the water which crosses the membrane cannot be *greater* than that in the solution (C_o) from which the net movement occurs. C_m can usually be calculated from known rates of flow. If C_m is greater than C_o, or if the net movement of the ion is not affected by the abolition of water movement, and if at the same time it takes place against the measured electro-chemical gradient, then the ion may be assumed to be actively transported.

B. ACTIVE TRANSPORT OF WATER

In the absence of a significant hydrostatic pressure gradient and of a net movement of solute, the driving force for passive water movement is proportional to the osmotic pressure gradient across the membrane. If there is a net movement of solute in the same direction as the water movement, then solute drag may provide an additional force. The importance of this factor has been demonstrated in artificial membranes (e.g. Durbin, 1960; Meschia and Setnikar, 1959). Meschia and Setnikar showed that solute drag due to the diffusion of urea across a collodion membrane could carry water against a large osmotic gradient. In a living membrane (roach gall-bladder) Diamond (1962) found that water could be carried by the passive diffusion of sodium chloride. Electro-osmosis is another example of a special case of solute drag (by an ion moving through a charged membrane). It is therefore necessary to test for the importance of drag effects before ascribing the movement of water against an osmotic gradient to active transport.

Simple qualitative tests follow the same lines as for solvent drag. The simplest is to replace the moving solute by one to which the membrane is impermeable and to observe the effect on the net water movement. Or, again, by calculation: if the water movement is due to solute drag, then the concentration of the solute in the water crossing the membrane cannot be *less* than that in the solution from which the net movement occurs. If it is, or if the water movement is not affected by the removal of the solute, and if it also takes place against the osmotic gradient, then active water transport can be assumed.

C. THE MOVEMENT OF WATER BY ACTIVE SOLUTE TRANSPORT

A most important advance in the understanding of the movement of water across living membranes comes from the study by Diamond (1962) of water transport across the wall of the roach gall-bladder. He showed that a net movement of water, against an osmotic gradient, is produced by the active transport of sodium chloride. His analysis based on irreversible thermodynamics, demonstrated that the mechanism of water transport is formally analagous to solute drag due to solute diffusion (co-diffusion).

It seems highly likely that a similar mechanism for water transport exists in many biological membranes. Although it is not possible to apply Diamond's analysis to other membranes until the required membrane parameters have been determined, strong circumstantial evidence for the presence of such a mechanism is provided if it is found that the

movement of water depends on the simultaneous movement of a solute, which is demonstrably actively transported. Whether this type of water transport should be regarded as active or not is obviously a matter of definition, but it is clearly distinguishable from active water transport *per se*.

III. Osmotic and Ionic Regulation in Aquatic Insects

Much is now known about the haemolymph composition in a large number of insects, although the analyses in many cases are far from complete (Buck, 1953; Duchâteau *et al.*, 1953; Clark, 1958; Wyatt, 1961; Sutcliffe, 1962b). However, there are few in which the ability to regulate the haemolymph composition has been investigated. Among the aquatic forms attention has been directed particularly towards larvae, especially of the Diptera—adult aquatic insects have been largely ignored. A comparison of the regulatory abilities of aquatic larvae found in fresh, brackish and saline waters is a first step towards the understanding of the underlying mechanisms of regulation in the insects as a whole.

A. Osmotic Regulation of the Haemolymph of Insects from Fresh Water

The usual procedure adopted to assess the regulatory capacity of an aquatic animal is to acclimatize specimens to as wide a range of external solutions of varying salinity as they will tolerate, and to compare the osmotic pressure of the medium (P_o) with that of the blood (P_i) after the new steady state value is attained. A high degree of regulation is indicated where $\Delta P_i/\Delta P_o$ approaches zero: the method also shows the external concentration at which regulation begins to break down, and also reveals the tolerance limits for the animal. The results of experiments of this kind on a number of freshwater insect larvae are shown in Fig. 1. It can be seen that the larvae of *Aëdes aegypti*, *Culex pipiens* and *Chironomus thumni thumni* exert a fine control over their internal osmotic pressure throughout a range of external solutions which extends far beyond the normal limits of a freshwater environment. However, when P_o exceeds the initial value of P_i, control breaks down and thereafter P_i closely follows changes in P_o, the two solutions becoming approximately iso-osmotic. In the larvae of *Limnephilus stigma* and *Anabolia nervosa* the regulatory capacity is not so highly developed: there is no regulation plateau, P_i, rising steadily with increasing values of P_o until the iso-osmotic state is reached, which is then maintained. In general, these larvae behave in a way which is typical of many freshwater

animals from other Phyla (see, for example, Beadle, 1943). These freshwater larvae do not survive well in solutions more concentrated than about 1 % sodium chloride, and this has also been found for the larvae of *Corethra* (Schaller, 1949) and *Helodes* (Treherne, 1954a).

Knowledge of the behaviour of adult freshwater insects is extremely scanty. Of the three species of *Sigara* (= *Corixa*) investigated by Claus (1937), the two freshwater forms, *C. distincta* and *C. fossarum*, showed a

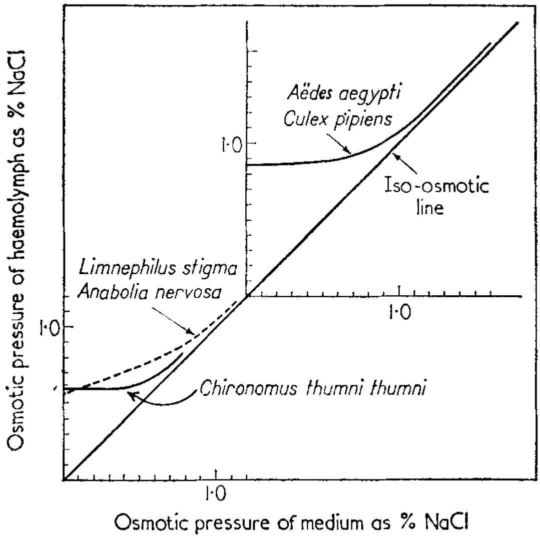

FIG. 1. The relationship between haemolymph osmotic pressure and that of the medium in various freshwater larvae. Where necessary, osmotic pressures have been recalculated as percent sodium chloride solutions (1% ≡ 171 mM/l). This and the following figures have been redrawn; where individual points are not given (for the sake of clarity) the curve shown was fitted to the points by eye. When considering the information in this and the following figures, due attention has been paid to the scatter in the results, for which the original papers must be consulted.

References: *Aëdes aegypti* and *Culex pipiens*—Wigglesworth, 1938; *Chironomus thumni thumni*—Neumann, 1961; *Anabolia nervosa* and *Limnephilus stigma*—Sutcliffe, 1961b.

gradual increase in haemolymph osmotic pressure as the external concentration was increased from 0·1 to 1·9 % sodium chloride. In view of the short duration of the experiments, however, it is doubtful if the measured haemolymph concentrations represent their true steady state values (Krogh, 1939).

B. INSECTS FROM BRACKISH WATER

Regulation curves for three species of insect larvae from brackish water are shown in Fig. 2. The first point of interest concerns their

tolerance limits. All can survive in fresh water—two of the species, *Limnephilus affinus* and *Chironomus aprilinus*, can also tolerate external salinities of over 2·5% sodium chloride—far beyond that of the freshwater forms. The third species, *Ch. halophilus*, has a wider range than its close relative, *Ch. thumni*, from fresh water. The second feature is that the regulation of P_i, which is reasonably efficient at the lower concentrations, is maintained at higher values of P_o so that the haemolymph becomes hypo-osmotic to the medium. Regulation breaks down soon

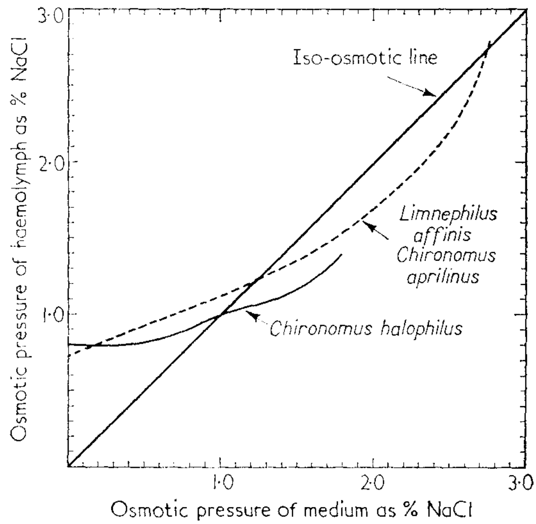

FIG. 2. The relationship between haemolymph osmotic pressure and that of the medium in various brackish-water larvae. Where necessary, osmotic pressures have been recalculated as percent sodium chloride solutions.

References: *Limnephilus affinis*—Sutcliffe, 1961a; *Chironomus aprilinus*—Sutcliffe, 1959; *Chironomus halophilus*—Neumann, 1961.

after, however, and P_i rises steeply. An important factor in the ability of *L. affinis* and *Ch. aprilinus* to survive in the higher salinities appears to be their ability to tolerate high haemolymph concentrations, which may rise to over 300% of the normal value.

A behaviour similar to that of *Ch. halophilus* has been described by Claus (1937) for the brackish-water corixid, *C. lugubris*. Again the question of inadequate equilibration time must be considered, but the possibility of a genuine difference in regulatory ability between this and the freshwater species is certainly not ruled out.

C. INSECT LARVAE FROM SALT WATER

As first shown by Beadle (1939) in the salt-water mosquito larva *Aëdes detritus*, these larvae are quite remarkable for their tolerance to very high external salinities and for their well-developed powers of hypo-osmotic regulation. Several larvae have now been examined and their regulation curves are shown in Fig. 3. The degree of regulation which they display is of a very high order and extends over a wide range

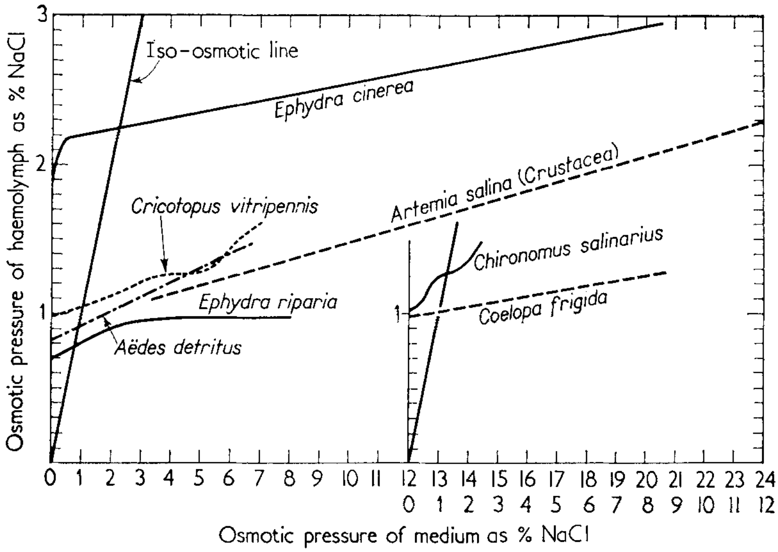

FIG. 3. The relationship between haemolymph osmotic pressure and that of the medium in various salt-water larvae. Where necessary, osmotic pressures have been recalculated as percent sodium chloride solutions. In the case of *Ephydra cinerea* the figures for the medium refer to the expression (density of medium—density of water) × 100, and are roughly equivalent to percent sodium chloride.

References: *Aëdes detritus*—Beadle, 1939; *Ephydra riparia*, *Cricotopus vitripennis*, and *Coelopa frigida*—Sutcliffe, 1960; *Chironomus salinarius*—Neumann, 1961; *Ephydra cinerea* —Nemenz, 1960a; *Artemia salina* (for comparison with *E. cinerea*)—Croghan, 1958a.

of external concentrations—the mean values of $\Delta P_i/\Delta P_o$ lie between 0·03 and 0·1 (with the exception of *Ch. salinarius*) and are comparable with (or better than) those for the freshwater forms over a much narrower range. The high degree of regulation may, perhaps, be correlated with the fluctuating conditions of their natural habitats—for example, salt marshes (*A. detritus*), rock pools, and stranded beds of decaying *Laminaria* (*Coelopa*).

Chironomus salinarius, which can tolerate external concentrations up

to about 5% sodium chloride (Neumann, 1961), appears to occupy an intermediate position between the majority of salt-water forms and those from brackish water. Part of its tolerance to the higher external concentrations must be due to its ability to withstand increases in the haemolymph concentration.

The larva of *E. cinerea* is of some interest as its natural habitat (the Great Salt Lake of Utah) has an equivalent salt concentration greater than 20% sodium chloride. The only other Metazoan found there is the crustacean *Artemia salina* (Nemenz, 1960a), and this displays a similar high degree of regulatory ability (Croghan, 1958a). The haemolymph osmotic pressure of *E. cinerea* is higher than most other aquatic insects, including its near relative *E. riparia*. Adaptation of the tissues to the higher haemolymph concentration may be an important factor in the ability of the animal to survive in the extremely high external concentrations, since the amount of osmotic work necessary to maintain the hypo-osmotic condition is reduced.

In dilute external solutions the salt-water larvae become hyperosmotic to the medium. Salt uptake mechanisms have yet to be demonstrated in these forms and it is possible that, due to the relative impermeability of their cuticles, a steady state was not achieved during the experimental periods.

D. THE IONIC COMPOSITION OF THE HAEMOLYMPH AND ITS REGULATION

1. *The normal composition*

Analyses of the inorganic constituents of the haemolymph of many aquatic insects are available in papers by Buck (1953), Clark (1958), Duchâteau *et al.* (1953), and a recent paper by Sutcliffe (1962b) deals specifically with this subject. From the wealth of data some general features appear to emerge (Sutcliffe, 1962b). In all aquatic species examined (with the exception of the larva of the lepidopteran, *Nymphula nymphaeta*) the sodium concentration is high (69–155 mM/l) and exceeds that of the potassium (2–31 mM/l) which is often quite low. There is no correlation between the sodium/potassium ratio and the diet of the insect. In the exopterygote insects the chloride concentration is also high (75–127 mM/l), but does not exceed that of sodium. In the Endopterygotes, on the other hand, the chloride concentration is much lower (8–50 mM/l) and large amounts of other anions are present. A few typical analyses are shown in Table I.

Another feature, which has important consequences for the mechan-

TABLE I

The ionic constitution of the haemolymph of some fresh water insects (from Sutcliffe, 1962b)

Order and species	Stage (larva or adult)	Haemolymph osmotic pressure as mM/l NaCl	Concentrations in mM/l							
			Na	K	Cl	Ca	Mg	HCO_3	Inorganic phosphate	Free amino acids (as glycine)
ODONATA										
Aeschna grandis	L	206	145	9	110	7·5	7·5	15	4	39
PLECOPTERA										
Dinocras cephalotes	L	163	117	10	111	—	—	—	6	39
COLEOPTERA										
Dytiscus marginalis	A	208	126	14	44	11·5	19	—	—	—
TRICHOPTERA										
Limnephilus stigma	L	106	83	14	10	—	—	10	12	41
LEPIDOPTERA										
Nymphula nymphaeta	L	154	40	29	31	—	—	—	—	—
DIPTERA										
Aëdes aegypti	L	142*	100†	4·2†	51·3	—	—	—	—	—
NEUROPTERA										
Sialis lutaria	L	170	109	5	31	7·5	19	15	10	85

In this and the following tables average values only are given for the sake of clarity; when considering the information contained in them due attention has been paid to the scatter in the results, for which the original papers must be consulted.

* Wigglesworth, 1938; † Ramsay, 1953

isms of osmoregulation and is in contrast with the situation found in many terrestrial insects, is that the haemolymph osmotic pressure is made up largely of electrolytes. Thus, as with most other animals, osmoregulation must depend on the underlying process of regulation of the ionic composition.

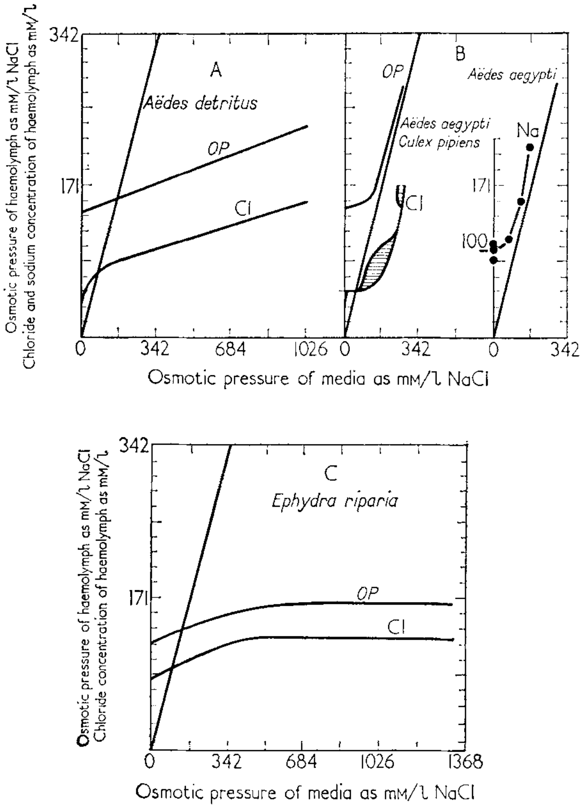

FIG. 4. The effect of increased concentrations of the medium upon the osmotic pressure (expressed as mM/l NaCl) and some of the ions of the haemolymph.

The media were various dilutions of sea water except in the case of the sodium data for *Aëdes aegypti*, where sodium chloride solutions were used. The spread in the chloride data for *Aëdes* is indicated by the cross-hatched area.

References: *Aëdes detritus*—Beadle, 1939; *Aëdes aegypti* and *Culex pipiens*—Wigglesworth, 1938; *Aëdes aegypti* (sodium data)—Ramsay, 1951, 1953a; *Ephydra riparia*—Sutcliffe, 1960.

2. *The regulation of ionic concentration*

The ability of aquatic insects to regulate the concentrations of individual ions in the haemolymph may be tested by varying the

concentration of the ion in the external medium. The regulation of sodium and chloride has been studied in animals acclimatized to external solutions of sodium chloride (or diluted sea water): regulation curves for a number of aquatic larvae are shown in Figs. 4 and 5. Although it is convenient to examine the regulation of the two ions separately, it must be remembered that the regulation of one may affect that of the other. A more elaborate experimental procedure would be required in order to test the independent regulation of the two ions.

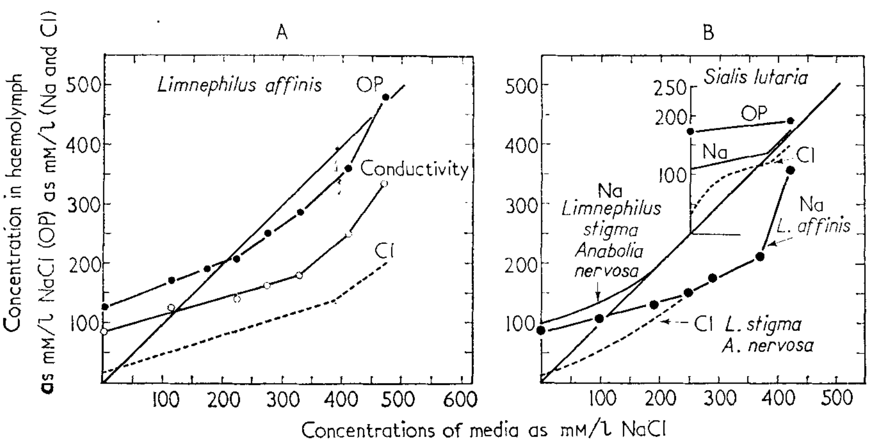

FIG. 5. The effect of increased concentrations of the medium upon the osmotic pressure (expressed as mM/l NaCl) and some of the ions of the haemolymph.

The media were various dilutions of sea water except in the case of *Sialis lutaria* where sodium chloride solutions were used.

References: *Limnephilus stigma* and *Anabolia nervosa*—Sutcliffe, 1961b; *Limnephilus affinis*—Sutcliffe, 1961a; *Sialis lutaria* (osmotic pressure)—Beadle and Shaw, 1950; *Sialis lutaria* (sodium and chloride)—Shaw, 1955b.

a. Sodium. The regulation curves for sodium in all the freshwater larvae examined (*A. aegypti*, *S. lutaria*, *L. stigma* and *A. nervosa*) follow closely those of the haemolymph osmotic pressure. It is thus apparent that under these conditions the regulation of the haemolymph osmotic pressure is dependent on the regulation of the electrolyte fraction of the haemolymph (since sodium is the major cation—see Table I). In the brackish-water larva, *L. affinis*, on the other hand, there is evidence of an additional regulation of the non-electrolyte fraction (Sutcliffe, 1961a; also see next section). The regulation of the sodium concentration is more effective than that of the osmotic pressure at the higher external concentrations and the haemolymph sodium is kept below the external sodium (Fig. 5).

b. Chloride. The regulation curves for chloride in several aquatic larvae (*L. stigma*, *A. nervosa*, from fresh water; *L. affinis* from brackish water; *E. riparia* from salt water) closely parallel those of sodium (or of total osmotic pressure, where sodium has not been measured), although the chloride concentration is maintained at a much lower level. In these larvae, and also in the larvae of *A. aegypti*, *A. detritus* and *C. pipiens*, the chloride concentration is kept below the external chloride at higher concentrations—although the larva of *Sialis* is unable to do this.

In general, the parallel behaviour of sodium and chloride suggests a similar regulation of these ions in the freshwater forms (except *Sialis*); at higher external concentrations, however, the haemolymph chloride is kept below that of the external medium, whereas the haemolymph sodium is always above the external sodium. Thus there is some indication of a degree of independent regulation of sodium and chloride—a view which is supported by the fact that there are certain larvae (*A. detritus*, *A. aegypti*, *C. pipiens* and *S. lutaria*) in which the chloride concentration parallels neither the sodium concentration nor the osmotic pressure over part of the external range (see Figs. 4 and 5).

c. Potassium. In the only aquatic larvae which have been examined (*A. aegypti* and *S. lutaria*) the regulation of the haemolymph potassium concentration is highly efficient. In *A. aegypti* the mean potassium concentration for larvae reared in distilled water is 3·1 mM/l. This is increased to 4·2 mM/l in an external solution of 1·7 mM/l potassium chloride, but is only raised to 5·7 mM/l in an external solution containing as much as 85 mM/l potassium (Ramsay, 1953a). *Sialis* larvae can maintain a relatively constant haemolymph potassium concentration (5mM/l) in external solutions up to 34 mM/l potassium chloride—at higher concentrations the blood potassium rises but the larvae can survive in solutions of 85 mM/l potassium chloride for 2 weeks (Shaw, 1955b). There is no reason to suppose that the regulation of the potassium concentration is not largely (if not completely) independent of the regulation of other haemolymph cations.

IV. The Mechanisms of Osmoregulation in Aquatic Insects

A. Regulation of the Non-Electrolyte Fraction of the Haemolymph

Evidence for the regulation of the non-electrolyte fraction of the haemolymph in the brackish-water larva of *Limnephilus affinis* has already been mentioned (see p. 327). In this larva the difference between

the total osmotic pressure and the sodium concentration (also the total electrolyte concentration, as measured by the haemolymph conductance) gradually increases as the external concentration is raised, until the breakdown of regulation occurs. It would appear that osmotically-active substances (presumably organic molecules) are mobilized and added to the haemolymph (Sutcliffe, 1961a). This additional regulatory mechanism may be concerned with the maintenance of water balance at the higher external salinities (Sutcliffe, 1961a, and see Section IV, D.).

In freshwater insects acclimatized to saline solutions, there is no evidence of the regulation of the non-electrolyte fraction, since changes in the total osmotic pressure are closely paralleled by changes in the sodium concentration (see p. 327). It is possible, however, that regulation of the non-electrolyte fraction may be important in normal animals. Wigglesworth (1938) observed that if the larvae of *Aëdes aegypti* are starved in distilled water the haemolymph chloride falls to about one-sixth of its initial value, whereas the total osmotic pressure is scarcely affected. Chloride variations without corresponding changes in the osmotic pressure have also been found in *Sialis* larvae (Beadle and Shaw, 1950), in *Aeschna* and *Libellula* larvae (Schoffeniels, 1950) and *Helodes* larvae (Treherne, 1954a). Unfortunately, in these experiments estimations of sodium (or conductivity) were not made; it is therefore not known to what extent the changes in chloride concentration represent changes in the total electrolyte content of the haemolymph. In view of the high sodium concentration found in freshwater forms (see Table I), it is possible that changes in the chloride concentration are compensated by changes in the remaining anion fraction—which is presumably also largely organic in nature (Shaw, 1955b; Sutcliffe, 1962b).

The assessment of the importance of, for example, changes in the haemolymph amino-acid content (nymphs of *Aeschna* and *Libellula*, and adult *Dytiscus*—Schoffeniels, 1960; *Drosophila* larvae—Zwicky, 1954), and of haemolymph non-protein nitrogen (*Sialis* larvae—Beadle and Shaw, 1950), in relation to the chloride concentration, must await full haemolymph analyses for the different experimental conditions.

B. THE RÔLE OF THE EXCRETORY SYSTEM

The aquatic insects, like terrestrial ones, possess an excretory system consisting of a number of Malpighian tubules which open into the alimentary canal at the junction of the mid-gut and the hind-gut. The Malpighian tubules are closed secretory tubules composed of a single layer of epithelial cells in which a fluid secretion, containing both organic

and inorganic substances, collects. The clear fluid produced in the tubules discharges into the hind-gut and is passed down to the rectum, from which it is periodically released to the exterior. Observations of the excretory process in the mosquito larva (Wigglesworth, 1938; Ramsay, 1950) reveal that the fluid is normally unmixed with mid-gut contents, although occasionally a small part of these is included. Clear uncontaminated rectal fluid can also be readily collected from many other aquatic insects (Shaw, 1955b; Sutcliffe, 1961a, b, 1962a).In the mosquito larva it is probable that some of the Malpighian tubule fluid passes forward into the mid-gut, later to be reabsorbed again by way of the mid-gut caeca (Wigglesworth, 1933b; Ramsay, 1953a).

The Malpighian fluid as discharged into the hind-gut may be regarded as the primary excretory fluid. Since it is known that the rectal fluid may be quite different in composition from this fluid, it follows that it is modified into its final form by the action of the hind-gut epithelium. The composition of Malpighian tubule fluid has been examined after collection by direct puncture in a few aquatic insects (Ramsay, 1951, 1953b). More usually, primary excretory fluid has been collected in the hind-gut just posterior to the point of entry of the tubules (intestinal fluid—Boné and Koch, 1942; Ramsay, 1950, 1951, 1953a). In the mosquito larva it appears that this fluid consists of Malpighian fluid relatively unchanged or uncontaminated by mid-gut contents—thus the composition of the fluid (total O.P. and Na concentration) is not significantly different from that collected after isolation from the mid-gut by ligaturing; and the sodium concentration of the fluid does not differ much from that of fluid collected directly from the tubule lumen (Ramsay, 1951).

1. *The composition of the primary excretory fluid*

Analyses of the Malpighian tubule fluid in three aquatic insects are shown in Table II (Ramsay, 1953a, b). In *Aëdes aegypti* larvae, reared in distilled water, the tubule fluid is usually slightly hypo-osmotic to the haemolymph, and this condition is maintained in larvae where the haemolymph osmotic pressure has been increased. Thus in larvae from 128 mM/l and 171 mM/l sodium chloride solutions the mean tubular osmotic pressure is 93% and 95% of that of the haemolymph respectively (Ramsay, 1951). The composition of the two fluids, however, is quite different.

It would appear that differences in chloride concentration are not marked. Boné and Koch (1942) found that in the larvae of *Limnophilus flavicornis* and *Chironomus plumosus* (both acclimatized to a 0·01% sodium chloride solution) the tubular chloride is approximately the

TABLE II

The composition of the primary excretory fluid, compared with that of the haemolymph in some aquatic insects

Species	Fluid	Concentrations in mM/l			Na ratio	K ratio	P.D. across tubule wall (mv)	Reference
		Na	K	Osmotic pressure ≡ NaCl soln.	$\frac{C_T}{C_H}$	$\frac{C_T}{C_H}$		
Aëdes aegypti (larvae from distilled water)	Haemolymph (C_H)	87	3	138	0·28	29·3	+21	Ramsay, 1950; 1953a, b
	Intestinal fluid (C_T)	24	88	130				
Dytiscus marginalis (adult)	Haemolymph (C_H)	140	5	—	0·31	31·1	+22	Ramsay, 1953b
	Tubule fluid (C_T)	44	156	—				
A Tabanid larva	Haemolymph (C_H)	151	5	—	0·17	32·0	—	Ramsay, 1953b
	Tubule fluid (C_T)	25	160	—				

same as that of the haemolymph. But with respect to sodium and potassium concentrations large differences are revealed (Table II). The potassium concentration is high in the tubule fluid and low in the haemolymph, whereas the reverse is true for sodium.

The ratio of potassium in the tubule to potassium in the haemolymph is rather constant in the three aquatic species examined, so is the sodium ratio. It may be noted that the divergence of these ratios from unity is rather greater in the aquatic than the terrestrial forms (see Ramsay, 1953b and p. 362). This is not due, however, to profound differences in tubular fluid composition between aquatic and terrestrial forms (indeed, they are rather similar), but to the fact that in the aquatic insects the haemolymph sodium tends to be higher, and the potassium lower, than in the terrestrial forms.

The Malpighian tubule fluid cannot be elaborated by a simple physical process, such as filtration or diffusion. Since the fluid is being continually produced and discharged, there must be a net flux of all the components of the fluid (including water) across the tubule wall into the lumen. The net flow of potassium takes place against the electrochemical potential gradient (Ramsay, 1953b). The remote possibility that the potassium flow is due to solvent drag (see Section II) can be readily eliminated. The concentration of potassium in the tubule fluid must be the same as that in the water crossing the tubule wall (C_m), and this clearly greatly exceeds that in the haemolymph (C_o). Since $C_m \gg C_o$, solvent drag cannot be an important factor (Section II). Thus active transport of potassium across the tubule wall is firmly established (Ramsay, 1953b).

The important consequences of Ramsay's demonstration of the active transport of potassium will not be pursued further in this Section—a more detailed account of the mechanism of excretion is reserved for Section VI, B. which deals with the terrestrial insects.

Transfer of sodium into the tubule may take place passively. There is a small electro-chemical gradient in favour of passive movement (Ramsay, 1953b), but whether this is sufficient to sustain the net sodium flux by diffusion alone is less certain. The flow might be increased by solvent drag—or, possibly, through a transport system working down the gradient (see Ramsay, 1955b).

2. *Modification of the primary excretory fluid*

In freshwater insects the primary excretory fluid is changed very substantially in the hind-gut before it is discharged. It is generally assumed that these changes result from the activity of the rectal epithe-

TABLE III

The composition of the rectal fluid of some fresh water insects

Order and Species	Stage (larva or adult)	Osmotic pressure ≡ mM/l NaCl	Concentrations in mM/l					Reference
			Na	K	Cl	NH_4	HCO_3	
DIPTERA								
Aëdes aegypti	L	12	4	25	—	—	—	Ramsay, 1950, 1953a
NEUROPTERA								
Sialis lutaria	L	59	12	4	0	100	91	Shaw, 1955b ; Staddon 1955 ; Sutcliffe, 1962c
TRICHOPTERA								
Limnephilus stigma	L	61	—	—	0	—	—	Sutcliffe, 1961b, 1962c
Anabolia nervosa	L	43	—	—	0	—	—	Sutcliffe, 1961b, 1962c
Limnophilus flavicornis	L	—	—	—	0·8	—	—	Boné and Koch, 1942
Phryganea striata	L	—	—	—	—	37–59	—	Staddon, 1962
PLECOPTERA								
Dinocras cephalotes	L	75	—	—	—	—	—	Sutcliffe, 1962c
COLEOPTERA								
Dytiscus marginalis	A	19	—	—	—	—	—	Sutcliffe, 1962c
Colymbetes fuscus	A	101	—	—	—	—	—	Sutcliffe, 1962c
Acilius sulcatus	A	29	—	—	—	—	—	Sutcliffe, 1962c
HEMIPTERA-HETEROPTERA								
Notonecta glauca	A	73	—	—	—	75	75	Staddon, 1962

lium, although it is possible that other parts of the hind-gut also participate. The composition of the rectal fluid of a number of freshwater forms is shown in Table III. It may be noted, in passing, that the fluid collected from the rectum (upon which these measurements have been made) may not have exactly the same composition as the fluid which is finally discharged from the anus. This almost certainly accounts for the rather high values of sodium and potassium found in the rectal fluid of *A. aegypti* and *S. lutaria* (Ramsay, 1953a; Shaw, 1955b). The available analyses of the rectal fluid cover rather a small range of aquatic insects and are far from complete, but several general features seem to emerge. The first point is that although the rectal fluid is always hypo-osmotic to the haemolymph, it is not generally very dilute—the osmotic pressure may be as much as 60% of that of the haemolymph. Despite this, the major monovalent inorganic ions of the haemolymph (Na, K and Cl) are present in low concentration—particularly in the case of chloride. There is, therefore, no serious loss of these ions through the excretory system.

Many aquatic insects are known to produce ammonia as their main nitrogenous excretory product (Staddon, 1955) and, from the few measurements of ammonia concentration in the rectal fluid which have been made (in *S. lutaria* and *Phryganea striata*), it would appear that the high osmotic pressure is due to the presence of this substance, probably in combination with bicarbonate (Shaw, 1955b).

3. *Reabsorption in the rectum*

There can be little serious doubt that the modification of the primary excretory fluid within the rectum involves the reabsorption of ions. This is clearly indicated by a comparison of the composition of the primary excretory fluid (intestinal fluid) with that of the rectal fluid in the larvae of *A. aegypti* and *L. flavicornis* (Table IV). A reduction in the concentration of sodium and potassium in the mosquito, and of chloride in the caddis larvae, is clearly evident. That this is of general occurrence in aquatic insects cannot yet be claimed, but there is a little evidence to support it. In *Dytiscus* the tubular potassium is high, and the sodium relatively so (Table II): but the total solute concentration in the rectal fluid is low (Table III). The potassium concentration in the rectal fluid of *Sialis* is low—yet higher concentrations were found in fluid collected from the more anterior part of the hind-gut (Shaw, 1955b). Direct evidence for the reabsorption of ions in the rectum of a terrestrial insect (Phillips, 1961) will be presented later (see p. 374)—and this adds weight to the more circumstantial evidence from the aquatic forms.

An alternative view, that dilution of the rectal fluid is brought about

by the addition of water, cannot be completely ruled out, although it is not in accord with Wigglesworth's (1933b) observations on the mosquito larva. He found, to the contrary, that even in fresh water some rectal reabsorption of water appeared to take place.

Speculations as to the mechanisms involved in the reabsorption of ions by the rectum are a little premature. However, if it is conceded that the reabsorption of sodium, potassium and chloride is highly probable, then some predictions can be made. A mean value of 24mV was found for the

TABLE IV

The reabsorption of ions in the rectum of aquatic larvae

Species	Fluid	Concentrations in mM/l				Reference
		Osmotic pressure ≡ NaCl soln	Na	K	Cl	
Aëdes aegypti	Intestinal fluid	101	24	88	—	Ramsay, 1950, 1953a
(distilled water larvae)	Rectal fluid	12	4	25	—	
Limnophilus flavicornis	Intestinal fluid	—	—	—	7·5	Boné and Koch, 1942
(larvae from 0·01% NaCl)	Rectal fluid	—	—	—	0·8	

potential difference across the rectal wall in *Sialis* larvae (haemolymph negative to the lumen—Shaw, 1955b). The reabsorption of sodium and chloride to the levels recorded in the rectum would require the active transport of both ions. The electro-chemical gradient favours the passive reabsorption of potassium but only down to a rectal concentration of about 2 mM/l—this is lower than the recorded value but may be higher than that in the fluid which is finally discharged.

The problem of the appearance of ammonium and bicarbonate ions in the rectal fluid remains unsolved. It is possible that these ions are present in the original Malpighian tubule secretion, although the combined concentrations of potassium and sodium also present may seem to preclude this. It may be considered more likely that the ammonia, at least, is secreted directly into the rectum, possibly being exchanged for

sodium and potassium during their reabsorption. The ammonification of the rectal fluid in an aquatic insect may parallel the similar process which takes place in the distal tubules of some vertebrate kidneys (see, for example, Smith, 1956).

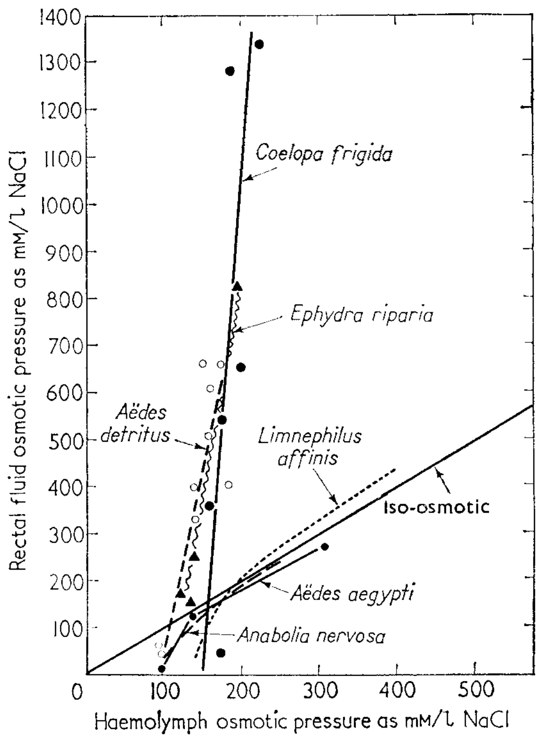

FIG. 6. The relationship between the osmotic pressure of the rectal fluid and that of the haemolymph for aquatic larvae from fresh, brackish, and salt water.

References: *Aëdes aegypti* (fresh water)—Ramsay, 1950; *Anabolia nervosa* (fresh water)—Sutcliffe, 1961b; *Limnephilus affinis* (brackish water)—Sutcliffe, 1961a; *Aëdes detritus* (salt water)—Ramsay, 1950; *Ephydra riparia* and *Coelopa frigida* (salt water)—Sutcliffe, 1960.

4. *Regulation of the rectal fluid composition*

There is clear evidence from a number of aquatic insects that the composition of the rectal fluid is regulated in relation to that of the haemolymph. First we may examine how changes in the haemolymph osmotic pressure, induced by the acclimatization of the animals to external solutions of varying salinity, are reflected by changes in the osmotic pressure of the rectal fluid. Figure 6 illustrates results which have been obtained on aquatic larvae from fresh, brackish and salt water.

The total osmotic pressure of the rectal fluid of the freshwater larvae (*A. nervosa* and *A. aegypti*) rises steeply as the haemolymph concentration increases, and becomes approximately iso-osmotic with it when the haemolymph osmotic pressure has risen to a value 40–50% above the normal: thereafter the two fluids remain roughly iso-osmotic. The brackish-water larva (*L. affinis*) displays a similar behaviour, except that at the higher haemolymph concentrations the rectal fluid becomes slightly but significantly hyper-osmotic to the haemolymph—a condition which is maintained over a wide range of the higher concentrations.

The larvae from salt water (*A. detritus*, *E. riparia* and *C. frigida*) stand out clearly from the other forms. In fresh water, *A. detritus* is able to produce a hypo-osmotic rectal fluid (Ramsay, 1950); but in their natural environment the rectal fluid of the salt-water larvae is very concentrated, often reaching a value of over four times that of the haemolymph osmotic pressure (Ramsay, 1950; Sutcliffe, 1960). Further, the control of the rectal fluid osmotic pressure is very sensitive to small changes in the haemolymph concentration. In some aquatic larvae, under the same experimental conditions, the concentration of the main inorganic ions in the rectal fluid has also been measured and can be related to their concentration in the haemolymph, as shown in Fig. 7.

The changes in sodium concentration of the rectal fluid of *A. aegypti* larvae parallel those of the osmotic pressure and this ion is the major cation of the rectal fluid of larvae from sodium chloride solutions (Ramsay, 1950, 1953a). The larva of *S. lutaria* shows similar changes in the rectal fluid sodium.

With regard to the excretion of chloride, there is evidence of species differences in the larvae of *L. affinis*, *A. nervosa* and *S. lutaria*. In *L. affinis*, from tap-water, the rectal fluid chloride is extremely low; but it rises steeply as the haemolymph chloride increases, reaching a value some three times that of the haemolymph level. In *A. nervosa* this is less well-marked—the rectal fluid chloride can exceed that of the haemolymph, but not to the same extent as in *L. affinis*. In *S. lutaria* the rectal fluid chloride never exceeds that of the haemolymph, and until the chloride concentration in the latter reaches a level of about 70 mM/l the rectal fluid concentration remains quite low. Attention has already been drawn to the differences in the degree of regulation of the haemolymph chloride in the aquatic larvae (see Figs. 4 and 5). The obvious correlation between this and their ability to concentrate chloride in the rectal fluid needs no further comment.

Potassium can also be concentrated in the rectal fluid. The regulation

of the haemolymph potassium has been seen to be highly efficient (p. 328); small increases in the haemolymph potassium lead to large increases in the rectal fluid potassium in the larvae of *A. aegypti* (Ramsay, 1953a) and of *S. lutaria* (Shaw, 1955b). The rectal fluid potassium may greatly exceed that of the haemolymph. In *S. lutaria* the rate of rectal fluid production is maintained even in iso-osmotic potassium chloride solutions—in contrast to solutions of sodium chloride—so that the rate of potassium excretion continues at a high level (Shaw, 1955b).

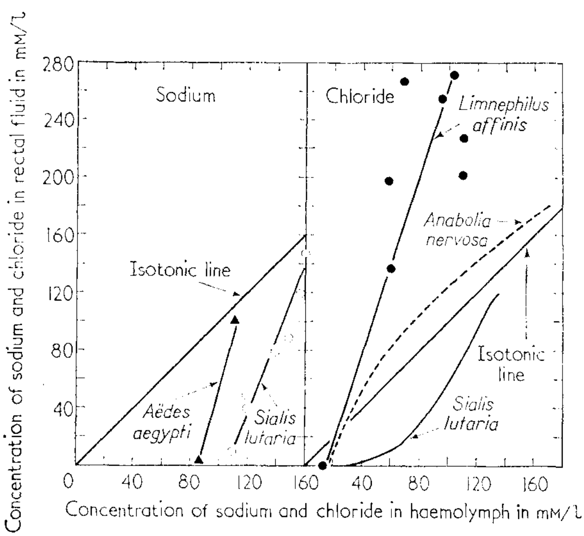

FIG. 7. The relationship between the sodium and chloride concentrations of the rectal fluid and their concentrations in the haemolymph in some aquatic larvae.

Reference: *Aëdes aegypti*—Ramsay, 1953a; *Sialis lutaria*—Shaw, 1955b; *Limnephilus affinis*—Sutcliffe, 1961a; *Anabolia nervosa*—Sutcliffe, 1961b.

5. *The mechanism of control of the rectal fluid composition*

The part played by the Malpighian tubules and by the rectum in the elaboration of the rectal fluid may now be considered. The first question concerns the relative rôle of the two organs in determining the final osmotic pressure of the rectal fluid. In the freshwater larva of *A. aegypti* the position is clear. Ramsay's measurements of the osmotic pressure of the Malpighian tubule fluid have already been given (p. 330)—the fluid is never more than slightly hypo-osmotic to the haemolymph, so the Malpighian tubules can play no more than a very minor rôle in osmotic regulation. In fresh water the osmotic work of dilution of the rectal

contents is performed in the rectum (Ramsay, 1950). In saline solutions, where the rectal fluid remains iso-osmotic with the haemolymph, no change in osmotic pressure is effected by the rectum—although the volume of the rectal fluid is decreased (Wigglesworth, 1933c).

In the salt-water larva, *A. detritus*, an analagous situation exists. The intestinal fluid is somewhat hyperosmotic to the haemolymph, but the bulk of the osmotic work of concentration is carried out in the rectum (Ramsay, 1950). By analogy with the situation in the terrestrial insects (Phillips, 1961, and see Section VI, B.) it is most likely that the concentration of the rectal fluid is achieved by the reabsorption of water in the rectum against an osmotic gradient.

TABLE V

The composition of the haemolymph, intestinal and rectal fluids of the larvae of *Aëdes aegypti* acclimatized to NaCl solutions (from Ramsay, 1951, 1953a)

External Medium	Concentrations in mM/l				
	Haemolymph Na	Intestinal fluid Na	Rectal fluid Na	Intestinal fluid K	Rectal fluid K
Distilled water	87	24	4	88	25
85 mM/l NaCl	113	71	100	90	18
128 mM/l NaCl	156	106	—	—	—
171 mM/l NaCl	209	205	—	—	—

The relative rôles of the Malpighian tubules and rectum in the regulation of the sodium concentration of the rectal fluid in *A. aegypti* are also revealed by analyses of the fluid from the intestine and rectum of these larvae adapted to external solutions of sodium chloride (Ramsay, 1950, 1953a; Table V). The Malpighian tubules respond to an increase in haemolymph sodium by secreting a fluid which contains more sodium than normal; at high haemolymph concentrations the fluid becomes practically isotonic in sodium. The sodium in this fluid may be further concentrated in the rectum—in contrast to the normal reabsorption—and despite the fact that potassium absorption takes place in the normal manner. However, in view of the fact that water is also extensively reabsorbed under conditions of high haemolymph sodium, it is highly

likely that some sodium reabsorption still occurs, despite the fact that its concentration in the rectum is increasing.

The regulation of potassium concentration in the rectal fluid of *A. aegypti* takes place in much the same manner (Ramsay, 1953a; and Table VI). The Malpighian tubules respond to a small increase in haemolymph potassium by increasing the concentration of the tubule fluid. Reabsorption in the rectum is curtailed but not completely abolished and the potassium concentration of the intestinal fluid falls to some extent in the rectum. Sodium reabsorption takes place in the normal manner.

TABLE VI

The composition of the haemolymph, intestinal and rectal fluids of the larvae of *Aëdes aegypti* from distilled water and from 85 mM/l KCl (from Ramsay, 1953a)

External Medium	Concentrations in mM/l				
	Haemolymph K	Intestinal fluid K	Rectal fluid K	Intestinal fluid Na	Rectal fluid Na
Distilled water	3·1	88	25	24	4
85 mM/l KCl	5·7	138	90	23	14

The means by which regulation of the chloride concentration of the rectal fluid is effected is not known. Changes in the chloride concentration might be due to water reabsorption or chloride secretion in the rectum, or to an increase in the chloride concentration of the Malpighian tubule fluid. The only relevant observation is that of Boné and Koch (1942). They found that in the larva of *Limnophilus flavicornis*, acclimatized to a 0·1 % sodium chloride solution, the chloride concentration of the intestinal fluid was slightly, but significantly, greater than that of the haemolymph (which was not the case for larvae from more dilute solutions). This suggests that the Malpighian tubules may play more than a passive rôle in chloride regulation, but the matter clearly requires further investigation.

C. THE UPTAKE OF INORGANIC IONS

1. *The uptake of ions by freshwater Culicine larvae*

The fact that Culicine larvae can take up ions from very dilute solutions is well established. Koch (1938) and Wigglesworth (1938)

demonstrated the uptake of chloride by the larvae of *Culex*, *Aëdes*, and *Chironomus*, and Koch (1938) showed that the uptake mechanism in *Culex* and *Chironomus* was located in the anal papillae. These organs have also been shown to be the site of uptake of sodium and potassium in the larvae of *Aëdes aegypti* (Ramsay, 1953a; Stobbart, 1960). The anal papillae are also the main site for the exchange of phosphate (Hasset and Jenkins, 1951).

a. The mechanism of ion uptake by the papillae. The uptake of inorganic ions by these larvae takes place against a large concentration gradient, but in order to decide if their movement is due to active transport, a knowledge of the electro-chemical gradient and of the importance of solvent drag is necessary (see Section II). Solvent drag may arise where there is a large net movement of water, and in *A. aegypti* and *Corethra plumicornis* the anal papillae are also known to be the main site of osmotic water uptake (Wigglesworth, 1933a; Schaller, 1949) which, in *Aëdes*, Wigglesworth estimated to be not more than the volume of two papillae per h. This corresponds to about 0·02 μl water per h, since the papillae are roughly cylinders 0·5 mm long and 0·16 mm in diameter. Sodium deficient larvae take up sodium from a 2 mM/l NaCl solution at 8·7 mM/l of haemolymph/h (Stobbart, 1960), and as the haemolymph volume is about 0·8 μl, this amounts to 6·9 mμM of sodium per h. Thus the concentration (C_m) of sodium in the water entering the papillae is 6·9/0·02 = 345 mM/l: a value which vastly exceeds that of the external solution (C_o = 2 mM/l) and, hence, solvent drag is not an important factor in the uptake of sodium.

Treherne (1954b) found that potassium did not compete with sodium for the uptake: this suggests separate transport systems for these ions and also provides slight evidence in favour of their active transport. Recently (Stobbart, 1962) preliminary measurements of the potential difference between the haemolymph of starved larvae and the medium (containing 2 mM/l NaCl) were obtained for these circumstances: (*a*) larvae in a steady state with respect to the medium; (*b*) larvae with about 5 % of the haemolymph sodium removed by brief pretreatment with de-ionized water, and taking up sodium and probably chloride (Wigglesworth, 1938). For (*a*) the potential difference was about +10 mV (sign refers to haemolymph), for (*b*) about —3 mV at the start of the period of uptake (1 h) rising to about —30 mV later. The equilibrium concentration of sodium in the haemolymph for a potential difference of —30 mV is only 6·5 mM/l which may be compared with the actual concentrations of between 95 and 100 mM/l, thus active transport of sodium must occur. Possibly, in accordance with the evidence for independent mechanisms

in other fresh water animals (Krogh, 1939; Jørgensen *et al.*, 1954; Shaw, 1960), separate active transport mechanisms occur for sodium, potassium and chloride.

(*b*). *The properties of the sodium uptake mechanism.* Further knowledge of the sodium transport mechanism in *A. aegypti* larvae comes from studies of the rates of sodium exchange (sodium fluxes) between haemolymph and medium (Treherne, 1954b; Stobbart 1959, 1960). In

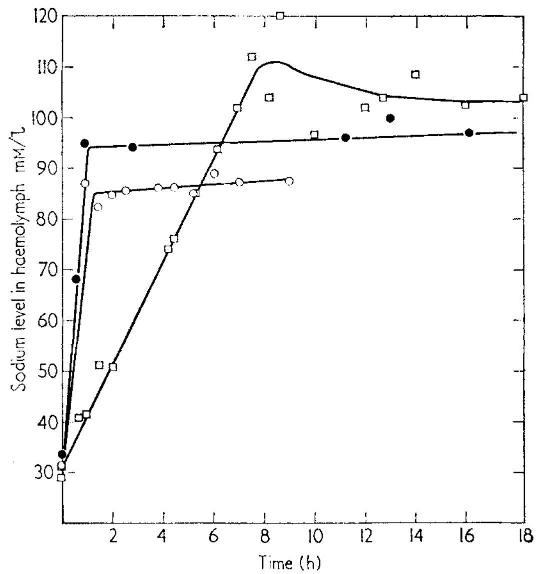

FIG. 8. The net uptake of sodium into the haemolymph by sodium deficient larvae of *Aëdes aegypti*. Open squares, starved larvae; open and solid circles, fed larvae (two experiments) (Stobbart, 1960).

starved larvae the steady state sodium exchange (measured with the isotopes ^{22}Na or ^{24}Na) is exponential, with a half exchange time ($T_{\frac{1}{2}}$) of about 63 h. The exchange involves all the haemolymph sodium, and the rate is the same as that for the whole larva, since a very large proportion of the body sodium occurs in the haemolymph. The rate of exchange corresponds to a sodium flux of between 1·0 and 1·2 mM sodium/l of haemolymph/h and is roughly the same in larvae acclimatized to media with a sodium concentration ranging from 2 to 8 mM/l. A very large proportion of the exchange—about 90%—takes place through the anal papillae (Treherne, 1954b; Stobbart, 1959). In fed larvae, the steady state exchange is increased by a factor of about 6·5, although there is no

noticeable difference in the haemolymph sodium concentration. The increased fluxes, which occur largely through the papillae, can be correlated with histological changes in the epithelial cells of these organs —in general, the cellular layer is much thicker in the fed animals (Stobbart, 1959).

By rearing larvae in a medium with a very low sodium concentration (1—2 μM/l) the haemolymph sodium can be kept down to about one-third of its normal value (sodium-deficient larvae). When these larvae are transferred to a solution containing 2mM/l sodium there is a rapid net uptake of sodium which proceeds at a roughly constant rate until the normal haemolymph level has been reached, after which net uptake practically ceases (see Fig. 8). The net uptake by fed larvae is also roughly 6·5 times as rapid as in the starved ones. During the net uptake period the sodium fluxes are many times greater than the steady state values, but fall back to normal at the completion of the uptake (Stobbart, 1960). The values of the sodium fluxes in normal and sodium-deficient larvae are shown in Table VII.

In recent experiments it has been found that normal *A. aegypti* larvae can maintain a steady state in an external medium containing as little as 6 μM/l sodium—a striking fact which may perhaps be correlated with the normal habitat of the larva—and the haemolymph sodium is only 5% below that of larvae from a 2 mM/l NaCl solution. The steady state fluxes are lower than in larvae from the more concentrated solution, but on increasing the external sodium concentration the initial sodium influx rises steeply. The sodium transport system appears to reach saturation at an external concentration greater than about 1 mM/l (Stobbart, 1962—Fig. 9). In this respect the sodium uptake mechanism resembles that found in the fresh water Crustacea (Shaw, 1959a, b; 1961). The net uptake rate of these larvae when transferred to 2 mM/l NaCl is of the same order as that of the sodium-deficient larvae. It is therefore probable that the removal of only 5% of the haemolymph sodium is enough to bring the sodium pump into full activity.

Koch (1954) found that anticholinesterases specifically inhibited the net transport of sodium in the larvae of *Chironomus*. However, physostigmine sulphate was without effect on the net sodium transport in *Aëdes* larvae (Stobbart, 1960).

c. The interpretation of the sodium flux measurements. In *A. aegypti* larvae, acclimatized to a medium containing 2 mM/l sodium, the sodium efflux to the medium is at least twice that found if the medium is replaced by distilled water. In addition, during net uptake of sodium by sodium-deficient larvae, the efflux is clearly not independent of the simultaneous

TABLE VII

Sodium fluxes in the house of *Aëdes aegypti*

(Data from Stobbart 1959, 1960)

State of larvae	*Larvae deficient in sodium*			*Normal larvae*		
	Rate of net uptake of sodium mM/l/h	Initial rate of influx of sodium mM/l/h	Initial rate of efflux of sodium mM/l/h	Steady-state flux of sodium mM/l/h	$T_{\frac{1}{2}}$ for steady-state exchange of sodium h	Net loss of sodium into flowing distilled water mM/l/h
Fed	39·5	88·5	49	7·15–8·84	9·1–10·0	—
Fed	50·5	120	69·5			
Starved	8·35	17·5	9·11	1·05–1·13	63·5	Approx. 0·5
Starved	8·5	14·4	5·85			
Starved	9·36	18	8·66			
Starved	6·96	10	3·04			

influx—see Table VII and Fig. 9. It follows, therefore, that the efflux is not just a measure of the passive sodium loss rate, but includes an exchange component (analagous to exchange diffusion as postulated by Ussing, 1947, 1948). With this in mind it has been suggested (Stobbart, 1959, 1960) that the movements of sodium can best be interpreted in

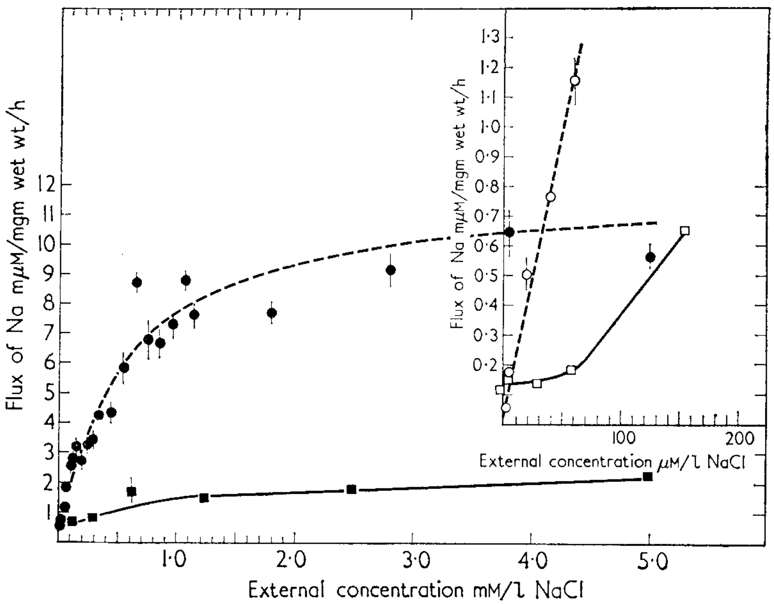

FIG. 9. The influence of external sodium concentration upon influx and efflux of sodium in starved larvae of *Aëdes aegypti*. The larvae were pretreated with de-ionized water (10 larvae/20 ml for 20 h). The balance point where influx=efflux is shown in the inset. Temperature, 28°C. Circles=influx; squares=efflux; the vertical lines show the extent of the standard deviations. The curve through the values for influx was calculated according to the Michaelis equation (cf. Shaw, 1959a) for a maximum influx of 12 mμM/mgm wet weight/h, and an external concentration for half-saturation of 0·55 mM/l Na (Stobbart, 1962).

terms of a sodium pump working in conjunction with an exchange diffusion mechanism (EDM) confined to a sodium-impermeable osmotic barrier in the papillae. It is supposed that during steady state exchange the pump splits off at the inner surface of the barrier sufficient sodium from the exchange diffusion mechanism to balance passive losses, the sodium on the EDM remaining free to exchange with sodium in the haemolymph or medium. Upon reaching the outer surface of the barrier the EDM takes up more sodium from the medium. In the fed larvae the

exchange but not the pumping is increased and this might be achieved by a synthesis of more EDMs, by an increase in their rate of movement, or possibly by an increase in enzymic catalysis of the exchange (Mitchell, 1954). During net uptake both the exchange and the pumping of sodium are increased, the extent to which this is done depending on the metabolic state of the larva.

It seems possible that the presence of an exchange diffusion mechanism allows an ion to be moved across an osmotic barrier which is relatively impermeable to the net passive movement of the ion. If this is so then the presence of such exchange systems may be a characteristic feature of many cell membranes. Exchange components in the fluxes of sodium and chloride ions are also a feature of the uptake mechanisms in some aquatic Crustacea (Croghan, 1958b; Shaw, 1959a, 1960; Bryan, 1960) and a similar exchange occurs in the uptake of phosphate by *Micrococcus pyogenes* (Mitchell, 1954).

2. *The uptake of ions by other freshwater insects*

The larvae of *Helodes minuta* and *H. marginata* (Coleoptera) are able to absorb chloride from dilute solutions. About half the net uptake takes place through the anal papillae, the rest, apparently, through the gut even when the external chloride is very low (Treherne, 1954a). Krogh (1939) showed that the nymphs of *Libellula* and *Aeschna* are able to absorb both sodium and chloride by independent mechanisms, uptake taking place most likely in the rectal chamber. Krawany (as reported by Boné and Koch, 1942) described silver-staining plaques on the ventral surface of the sixth abdominal segment of the caddis larva, *Limnophilus flavicornis*, and these have been provisionally identified as chloride absorbing organs by Boné and Koch. Sutcliffe (1961b) found relatively large sodium fluxes through the body wall in *Limnephilus stigma*.

It appears very likely that ion-absorbing organs of various kinds will be found in many different aquatic larvae, but in some, such organs appear to be absent. Beadle and Shaw (1950) could find no evidence for the uptake of chloride by *Sialis lutaria* in tap water. The lack of an uptake mechanism may well be associated with a very low permeability of the cuticle to ions. In *S. lutaria* the low permeability of the body surface to ions (Shaw, 1955a) allows the larva to withstand washing in flowing distilled water for several weeks. In *Limnephilus affinis* larvae the ability to take up chloride from tap water is also lacking. The haemolymph chloride concentration steadily falls when the larvae are kept in running tap water (Sutcliffe, 1961a).

3. *The uptake of ions through the gut*

The uptake of chloride through the gut in *Helodes* larvae has been mentioned. The larva of *Sialis lutaria* is also able to take up ions in this manner in more concentrated solutions (Shaw, 1955b). The gut is rather permeable to both water and salts. If the larvae are induced to drink the medium, by the removal of some of the haemolymph, sodium and chloride are both absorbed in approximately equal amounts and at a rate which is roughly proportional to the concentration of the medium. A small potential difference across the mid-gut wall (18 mV—haemolymph positive to the lumen) is consistent with the view that sodium is actively absorbed (Shaw, 1955a, b). In normal larvae in external solutions of 34 mM/l NaCl or more, some drinking occurs and the haemolymph sodium and chloride concentrations are maintained above those of the medium (Shaw, 1955b). In other freshwater larvae in saline solutions, in the brackish-water larva of *L. affinis*, and in the salt-water larvae there is strong evidence that drinking normally takes place (see next section). Although the uptake of salts through the gut in these forms has not been studied, it can scarcely be doubted that the gut provides a major route for salt entry (Beadle, 1939). Drinking plays an important part in the mechanism of salt and water balance in the salt-water larva of *E. cinerea* (Nemenz, 1960a). Nemenz (1960b) also claims that in a hypo-osmotic medium this larva can take up sodium by a transport mechanism located in the cuticle. This conclusion, which is based on tracer studies with ^{22}Na without measurements of the specific activity or of the sodium concentration of the medium and haemolymph, is difficult to accept. Nevertheless, the possibility that ion transport mechanisms, located elsewhere than in the gut and the excretory system, may play a part in ionic regulation in salt-water insects must not be overlooked. A thorough investigation of ion fluxes in these forms is highly desirable.

D. WATER BALANCE

Regulatory mechanisms for the maintenance of the ionic composition of the haemolymph must depend upon the effective control of the normal water content, and the processes of water balance and ionic regulation are closely related. In the freshwater insects water balance is maintained by the excretion from the rectum of a volume of fluid which is equal to that which enters through the cuticle due to osmosis, and through the gut as a result of drinking. In the salt-water larvae, on the other hand, water loss occurs not only by way of the rectal fluid but also by osmotic

withdrawal through the cuticle, and this must be balanced by an equal water uptake.

a. Freshwater insects. In the larva of *Sialis lutaria* the absolute permeability of the cuticle to water, as measured by the exchange of D_2O, is low (permeability constant = 0·018 cm/h at 20°C); although it appears high when compared with that of the majority of terrestrial insects

TABLE VIII

Rates of water loss in dry air at 20°C for some aquatic insects (from Holdgate, 1956)

Species	Stage	Mean rate of water loss ($mg/cm^2/h$)
DIPTERA		
Tipula sp.	larva	11·5
TRICHOPTERA		
Limnephilus sp.	larva	11
NEUROPTERA		
Sialis lutaria	larva	6·4
ODONATA		
Coenagrion puella	nymph	4·2
Anax imperator	nymph	0·9
HEMIPTERA		
Corixa punctata	adult	2·1
Notonecta obliqua	adult	2·9
COLEOPTERA		
Agabus bipustulatus	adult	0·9
Hydrobius fuscipes	adult	0·3

(Shaw, 1955a). By applying Wigglesworth's (1945) method for measuring the rate of loss of water in dry air, to a number of aquatic insects Holdgate (1956) found that there is a great variation in water permeability among the different species (see Table VIII). The aquatic larvae and the nymph of *Coenagrion* are much more permeable than the adult forms. The adult aquatic beetles *Agabus* and *Hydrobius* are especially noteworthy for their low permeability—a condition which is also found in the anisopterous dragonfly nymph, *Anax imperator*. The number of insects examined has been extended by Beament (1961b), who concludes that the more impermeable aquatic insects have the same order of permeability as the less waterproof terrestrial forms. The more permeable

aquatic forms lack the highly orientated lipid waterproofing mechanism of the terrestrial insects, over some part (or over the whole) of their cuticles (Beament, 1961b). In the larva of *Ephemera* the cuticle of the tracheal gills has the same permeability as that of the rest of the cuticle; in the nymph of *Coenagrion* the gills are slightly more permeable than the thoracic cuticle, and in *Sialis* larvae the cuticle of the gills and abdomen is several times more permeable than that of the thorax (Beament, 1961b). Thus some degree of regional differentiation of cuticular properties is indicated, but there is no evidence to suggest that the cuticle of the tracheal gills of these larvae is substantially more permeable than the abdominal cuticle to which they are attached. In the aquatic larvae with anal papillae, on the other hand, there is strong evidence that the papillae are regions of high water permeability compared with the remainder of the body surface. This has been demonstrated by ligaturing experiments in the larvae of *A. aegypti* and *Corethra plumicornis* (Wigglesworth, 1933b; Schaller, 1949).

In the larvae without anal papillae the relatively low absolute permeability of the cuticle to water restricts the osmotic water uptake. In the larva of *Sialis*, for example, calculation of the osmotic uptake from the D_2O permeability constant gave a value of only 4% of the body weight per day at 20°C—and this value was confirmed by measurement of the increase in body weight after blockage of mouth and anus (Shaw, 1955a). Even in the more permeable caddis larvae (see Table IX), determination of the osmotic uptake rate by the weighing method in three species, *Limnephilus affinis*, *L. stigma* and *A. nervosa*, gave a value of not more than about 7% of the body weight per day (Sutcliffe, 1961a). Since the caddis larvae and that of *Sialis* are much more permeable than many of the aquatic insects, it is reasonable to conclude that the osmotic water influx is generally of a low order. The larvae with anal papillae, however, stand apart. There is no doubt that the specially high water permeability of the papillae give rise to a much higher rate of water turnover. The osmotic uptake rate in the larvae of *A. aegypti* and *C. plumicornis* has been calculated from the observed rate of rectal fluid production and is shown in Table IX, together with the measurements on other aquatic larvae.

In the freshwater larvae which have been examined there is no evidence that the normal water intake is increased substantially by drinking. This is based on visual observations in *A. aegypti* and *C. plumicornis* (Wigglesworth, 1933b; Schaller, 1949), on the measurement of the D_2O flux after blocking the mouth in *Sialis* larvae (Shaw, 1955a), and on the correspondence between the osmotic uptake of water and the

TABLE IX

Estimates of osmotic uptake of water in fresh water larvae at 15–20°C

Larva	Osmotic uptake % body weight per day	Medium	Method of estimation	Site of penetration	Reference
Aëdes aegypti	33*	Tapwater	Visual	Mainly anal papillae	Wigglesworth, 1933c
Chaoborus plumicornis	19·2	Tapwater	Visual	Mainly anal papillae	Schaller, 1949
Sialis luturia	2·7–5·8	Tapwater	Weighing, excretory aperture blocked	General body surface	Shaw, 1955a
Sialis lutaria	4	Tapwater	Heavy water	General body surface	Shaw, 1955a
Limnephilus affinis *Anabolia nervosa* *Limnephilus stigma*	7	Tapwater	Regular collections of rectal fluid : weighing, excretory aperture blocked	General body surface	Sutcliffe, 1961a, 1962a

* Maximal estimate using Wigglesworth's estimate of rectal fluid production and a body weight of 1·66 mgm.

output of rectal fluid in the caddis larvae (Sutcliffe, 1961a, 1962a). It is possible, however, that drinking is a more important factor in the less permeable aquatic insects. Some of these are known to excrete ammonia (Staddon, 1955) and yet they may produce a relatively dilute rectal fluid (see, for example, the adult *Dytiscus*: Table III). Since the osmotic uptake is small, uptake of water through the gut may contribute substantially to the total water intake.

Even those larvae which normally drink very little behave differently when transferred to a saline solution. They all start drinking, often taking in large amounts of the solution (Wigglesworth, 1933c; Schaller, 1949; Shaw, 1955b; Sutcliffe, 1962a). If the saline solution is iso-osmotic with the haemolymph, or stronger, it often has an adverse effect on the epithelial lining of the gut, leading to separation of the cells and an increased permeability of the gut wall (Wigglesworth, 1933c; Sutcliffe, 1962a).

The larvae of *Sialis* can also be induced to drink large amounts of the medium if the haemolymph volume is reduced. This provides a useful way of introducing experimental solutions into the gut and studying their uptake. With an iso-osmotic mannitol solution no water uptake from the gut takes place; but if the solution also contains some sodium chloride, then water is absorbed together with the salt as an approximately iso-osmotic sodium chloride solution (Shaw, 1955a, b). Although there is no reason to suppose that water absorbed in this manner makes a significant contribution to the normal water intake in *Sialis*, it does suggest a mechanism by which water can be taken up in the gut of the salt-water forms—a mechanism which involves the movement of water due to active solute transport (see Section II).

In the freshwater insects the control of water balance is not fully understood. Both the rate of drinking and the rate of rectal fluid production are variable factors. In *Sialis* larvae a reduction in haemolymph volume leads to an increased water uptake by drinking but not to a reduction in the rate of excretion (Shaw, 1955b). On the other hand, an increase in haemolymph volume leads to a greater rectal fluid output in the larva of *A. aegypti* (Ramsay, 1953a). In a number of larvae (*A. aegypti*, *C. plumicornis*, *S. lutaria*, *A. nervosa*) it has been shown that acclimatization to an iso-osmotic saline solution leads to a very greatly reduced rectal fluid production (Wigglesworth, 1933c; Schaller, 1949; Shaw, 1955b; Sutcliffe, 1962a), an effect which is probably due, in *Sialis* larvae at least, to an increase in the sodium chloride concentration of the haemolymph, since it is not observed in iso-osmotic solutions of mannitol or potassium chloride.

b. Brackish and salt-water larvae. The brackish-water larva of *L. affinis* resembles the freshwater caddis larvae in many ways but can survive in much higher external salinities. Water balance in the higher salinities appears to be maintained in the following way: the salt water is swallowed at a strictly controlled rate (3–7% of the body weight per day) and without adverse effect on the gut. From the absorbed water some is lost by osmotic withdrawal through the body surface, since the haemolymph is slightly hypo-osmotic to the medium (Fig. 2), and the rest is lost in the rectal fluid which is produced at a steady rate (although at slower rate than when in fresh water (Sutcliffe, 1961a, 1962a). One feature of importance is the relatively high permeability of the cuticle to water and this may be correlated with the inability of the larva to maintain its haemolymph more than slightly hypo-osmotic to the medium (Sutcliffe, 1961a).

In the salt-water larvae it is most likely that water balance is maintained in a similar manner. It seems certain that uptake of water through the gut takes place in the larvae of *A. detritus* and *E. cinerea* (Beadle, 1939; Nemenz, 1960a; Stobbart, 1962) and that osmotic regulation is maintained by the excretion of a hyperosmotic rectal fluid (Ramsay, 1950; Sutcliffe, 1960). In these strongly hypo-osmotic larvae the cuticle appears to be relatively impermeable to water and salts, so that the rate of osmotic water loss is restricted (Beadle, 1939; Nemenz, 1960a; Stobbart, 1962). From a study of the rate of penetration of D_2O, Nemenz (1960b) concludes that the permeability of the cuticle of the larva of *E. cinerea* is similar to that of *Sialis* larvae; but from the data he presents this is clearly not the case. The exchange rate is rather slow and the D_2O permeability constant of the cuticle must be at least an order of magnitude smaller than in *Sialis*.

V. Ionic and Osmotic Regulation in Terrestrial Insects

A. The Ionic Composition of the Haemolymph of Terrestrial Insects in Relation to Diet

The haemolymph of a large number of terrestrial insects has now been analysed (Boné, 1944, 1947; Duchâteau *et al.*, 1953; Buck, 1953; Clark, 1958; Wyatt, 1961) though in many cases the analyses are not complete. Some typical examples are shown in Table X; some of the Dipteran larvae are not strictly terrestrial but are included here for comparison.

Boné suggested that a low Na/K ratio is a general characteristic of the

haemolymph of phytophagous insects, but later it became clear that this is only true in certain cases. Duchâteau *et al.* suggested that a high Na/K ratio (i.e. greater than unity) characterizes the more primitive insects, but that it may be lower in the more highly evolved forms, in relation to the ionic composition of their diet. At present it appears that low Na/K ratios are found in the Hymenoptera irrespective of their diet, in the phytophagous Lepidoptera and in some phytophagous Coleoptera; a low ratio has also been found in one Dipteran larva, one Heteropteran and in the Phasmid, *Dixippus morosus*—these all being phytophagous forms. There are, however, many phytophagous insects with a high Na/K ratio, although no exclusively carnivorous insect with a low one has yet been found. Sutcliffe (1962b) found that in the aquatic insects the Exopterygota have a high haemolymph chloride concentration, but in the Endopterygota it tends to be much lower. Further observations (Sutcliffe, 1963) suggest that the same may be true of the terrestrial forms. In many terrestrial insects the inorganic ions make a relatively small contribution to the total osmotic pressure of the haemolymph.

Table XI compares the Na, K, Ca, Mg and Cl content of the diet with the composition of the haemolymph and tissues in a number of terrestrial insects. If the ions are absorbed in the same proportion as they are present in the food there is no doubt that a high degree of regulation is required in order to maintain the normal haemolymph composition. Magnesium is often concentrated to a considerable extent in the haemolymph, and this is the case for calcium also in some insects: the reverse is often true for potassium. It may be noted that the two insects shown in Table XI which feed on leaves with a very low sodium content have unusually low levels of sodium in the haemolymph.

The effect of alterations in the diet upon the composition of the haemolymph has been studied in a few insects. Tobias (1948a) was unable to find a significantly increased haemolymph potassium concentration after feeding *Periplaneta americana* on lettuce leaves, which contain much potassium, although it was increased threefold by introducing a concentrated KCl solution into the gut. The tissue composition was also changed, and this will be discussed later (Section VI). Phillips (1961) found a significant increase in ionic concentration and total osmotic pressure of the haemolymph of *Schistocerca gregaria* when starved at 70% relative humidity for 5–6 days and given a saline solution to drink (the controls being starved at 100% R.H. and given tap water)—Table XIIA. Hoyle (1954) found a significant drop in the haemolymph potassium concentration in adults and nymphs of *Locusta*

TABLE X

The ionic constitution of the haemolymph of some terrestrial insects

Order and species	Stage (Adult, Larva, Pupa)	Osmotic pressure as mM/l NaCl	m equiv/l					Reference
			Na	K	Ca	Mg	Cl	
EXOPTERYGOTA								
ORTHOPTERA								
Schistocerca gregaria	A	211	108	11	—	—	115	Phillips, 1961
HEMIPTERA-HETEROPTERA								
Rhodnius prolixus	A	198	164	6	—	—	—	Ramsay, 1952
Triatoma megista	A	—	133	5	—	—	—	Boné, 1944
DERMAPTERA								
Forficula auricularia	A	207	96	13	—	—	90	Sutcliffe, 1963
PHASMIDA								
Dixippus morosus	A	160*	8·7	27·5	16·2	145	93	*Rouschal, 1940 Duchâteau *et al.*, 1953
ENDOPTERYGOTA								
COLEOPTERA (POLYPHAGA)								
Geotrupes stercorosus	A	—	119	16	17·8	49·8	—	Duchâteau *et al.*, 1953
Melolontha melolontha	A	—	113	6	15·3	41·3	—	Duchâteau *et al.*, 1953
Popillia japonica	L	281	20	12	16	40	19	Ludwig, 1951
Timarchia tenebriosa	L	—	1·6	46·9	72·2	158	—	Duchâteau *et al.*, 1953
Leptinotarsa decemlineata	L	—	5	56·8	82	178	—	Duchâteau *et al.*, 1953

TABLE X—*continued*

Order and species	Stage (Adult, Larva, Pupa)	Osmotic pressure as mM/l NaCl	m equiv/l					Reference
			Na	K	Ca	Mg	Cl	
NEUROPTERA								
Osmylus fulvicephalus	A	226	92	40	—	—	62	Sutcliffe, 1963
MECOPTERA								
Panorpa communis	A	193	94	38	—	—	34	Sutcliffe, 1963
LEPIDOPTERA								
Bombyx mori	L	137	14*	40*	24·5†	101†	21	*Tobias, 1948b †Duchâteau *et al.*, 1953 Buck, 1953
Samia walkeri	P	316*	3	42	18	64	10	Buck, 1953 *Barsa, 1955
Cossus cossus	L	—	18·4	35·4	51·5	48	—	Duchâteau *et al.*, 1953
HYMENOPTERA								
Apis mellifera	L	246*	10·9 5,10	30·5 24,45	18·2 8*	20·5 16*	33*	*Buck, 1953 Duchâteau *et al.*, 1953
Neodiprion sertifer	L	—	3	38	—	—	17	Sutcliffe, 1963
DIPTERA								
Tipula paludosa and T. oleracea	L	—	84	8·2	12·3	16·0	—	Duchâteau *et al.*, 1953
Gastrophilus intestinalis	L	249	175	11·5	5·7	32	14·8	Levenbook, 1950
Drosophila melanogaster	L	171	52	36	—	—	30	Croghan and Lockwood, 1960

TABLE XI

The ionic constitution of the diet in relation to that of the haemolymph and tissues of some terrestrial insects. A = adult ; L = larva ; tr = trace

Diet and insect tissue (haemolymph unless stated otherwise)		m equiv/l or /kg wet weight Na	K	Ca	Mg	Cl	Reference
Whole human blood		87	51·1	3·0	2·48	81·7	"Principles of Human Physiology", Starling 12th Edn., 1956
Whole rabbit blood		—	—	3·6	2·0	—	Clark and Craig, 1953
Rhodnius prolixus	A	164	6	—	—	—	Ramsay, 1952
Triatoma infestens	A	—	—	40·9	1·48	—	Clark and Craig, 1953
Triatoma megista	A	133	5	—	—	—	Boné, 1944
Whole horse blood		84·8	31·4	1·7	3·3	—	Duchâteau *et al.*, 1953
Gasterophilus intestinalis	L	175	11·5	5·7	32	14·8	Levenbook, 1950
Lettuce leaves		13	87·2	—	—	—	Tobias, 1948a
Periplaneta americana	A	113	25·6	—	—	—	Tobias, 1948a
Muscle	A	41	106	—	—	—	Tobias, 1948a
Nerve cord and sheath	A	103	178	—	—	—	Tobias, 1948a
		103*	180*	—	—	—	*Treherne, 1961a

Table XI—*continued*

Diet and insect tissue (haemolymph unless stated otherwise)		m equiv/l or /kg wet weight Na	K	Ca	Mg	Cl	Reference
Privet leaves		46·4	152·1	824·5	39·9	—	Duchâteau *et al.*, 1953
Dixippus morosus	A	8·7	27·5	16·2	142	—	Duchâteau *et al.*, 1953
Carrot leaves		25·6	176·9	214·5	35·6	—	Duchâteau *et al.*, 1953
Papilio machaon	L	13·6	45·3	33·4	59·8	—	Duchâteau *et al.*, 1953
Potato leaves		tr	144·5	128·6	85·9	—	Duchâteau *et al.*, 1953
Leptinotarsa (Coleoptera) *decemlineata*	A	3·5	65·1	47·5	188·3	—	Duchâteau *et al.*, 1953
Honey		4·7	13·1	2·7	1·8	—	Duchâteau *et al.*, 1953
Honey bee	L	10·9	30·5	18·2	20·5	—	Duchâteau *et al.*, 1953
Ribes grossulariae leaves		tr	249·1	271·2	53·6	—	Duchâteau *et al.*, 1953
Pteronidea ribesii (Hymenoptera)	L	1·6	43·4	17·5	60·5	—	Duchâteau *et al.*, 1953

migratoria migratorioides after about 15 h of starvation at 25°C. He suggested that falling haemolymph potassium might be one of the factors contributing to the greater activity of migrating locusts, as it increases the resting potential and tension developed in the leg muscles.

TABLE XII

The effect of alterations in the diet upon the ionic composition of the haemolymph

Insect, diet and Haemolymph	Osmotic pressure as mM/l NaCl	mM/l Na	K	Cl	Reference
		A			
Schistocerca gregaria					*300 mM/l NaCl
Tapwater to drink R.H. 100%					150 mM/l KCl
					30 mM/l Mg(acetate)$_2$
Haemolymph	211	108	11	115	30 mM/l Ca $(NO_3)_2$
S. gregaria					
Saline to drink R.H. 70%					
Saline*	—	300	150	450	Phillips, 1961
Haemolymph	271	158	19	163	
		B			
Drosophila melanogaster (Oregon K)					
Normal larvae on standard medium					
Haemolymph	171	52	36	30	
Selected larvae on 7% NaCl medium					
Haemolymph	199	90	37	67	Croghan and Lockwood, 1960
Selected larvae on 7% KCl medium					
Haemolymph	181	35	48	71	

In contrast, Ramsay (1955a) found that in *Dixippus* 96 h starvation only led to a small decrease in the potassium level.

Croghan and Lockwood (1960) studied the composition of the haemolymph in normal larvae of *Drosophila melanogaster* (Oregon K) and in a substrain selected to survive and develop on a medium containing 7% sodium chloride (Waddington, 1959). In the substrain the anal organs, which may be concerned with salt transport (Gloor and

Chen, 1950; Quintart, 1961), are larger than normal. Since the larvae are continually feeding on the medium considerable amounts of salt must enter the gut. In the larvae from the 7% NaCl medium the total osmotic pressure, sodium and chloride are significantly higher than normal (see Table XIIB), whereas the potassium is unchanged. The larvae are also able to survive on a medium containing 7% KCl; the haemolymph potassium and chloride are then maintained at a slightly higher level than normal whereas the sodium is significantly lower. The Na/K ratio is depressed below unity. It was found, contrary to expectation, that normal larvae can also survive on a medium containing 7% KCl.

In a study of excretion in *Rhodnius prolixus*, Ramsay (1952) observed the effect on the haemolymph potassium of increased potassium in the blood meal. After a normal meal the changes in haemolymph sodium and potassium are slight. When the potassium content of the meal is increased by a factor of about 1·6 (3 parts blood, 1 part 171 mM/l KCl) the insects show no ill effects despite an appreciable increase in haemolymph potassium in some cases; but when it is increased by a factor of about 2·8 (1 part blood to 3 parts KCl) large increases in haemolymph potassium are observed. The high potassium level appears to interfere with neuromuscular function and the insects generally die after a few hours.

There seems little doubt that ionic regulation is a function of some importance in the terrestrial insects, just as in the aquatic forms. Although the studies on regulation in relation to dietary changes are still rather incomplete, there seems a clear indication from a number of forms that regulatory ability is well developed and is effective in the face of quite substantial alterations to the normal intake of ions. In the terrestrial insects the task of performing the regulation falls largely on the excretory system.

B. THE RÔLE OF THE EXCRETORY SYSTEM

There can be no doubt of the fundamental rôle which the excretory system plays in maintaining both the osmotic pressure and the ionic composition of the haemolymph of terrestrial insects. Basically the excretory system resembles that of the aquatic insects—the Malpighian tubules produce a primary excretory fluid which is discharged into the hind-gut to become highly modified before its eventual elimination from the rectum. In both the stick insect, *Dixippus morosus* (Ramsay, 1955a), and the locust, *Schistocerca gregaria* (Phillips, 1961), there is no

evidence that a significant proportion of the Malpighian tubule fluid passes forwards to the mid-gut. On the other hand, because of the relatively low rate of rectal fluid excretion in many terrestrial forms, the rectal fluid is much more subject to contamination from mid-gut contents than in the aquatic insects, and it is only in fasting animals that the rectal fluid can be regarded as having been derived largely from the modified Malpighian tubule secretion. The excretory system may also be much more complicated than the relatively simple arrangement in an aquatic larva, such as that of *Aëdes aegypti* (Wigglesworth, 1933b). For example, there may be more than one kind of Malpighian tubule present (as in *Dixippus;* Ramsay, 1955a) or the individual tubules may show regional differentiation along their length (as in *Rhodnius;* Wigglesworth, 1931b). Perhaps one of the most striking examples of complication of Malpighian tubule structure is the cryptonephridial arrangement, common in the Coleoptera and in the larvae of the Lepidoptera, where the distal parts of the tubules are closely associated with the wall of the rectum (see Wigglesworth, 1953). The hind-gut, too, is very variable in structure, ranging from the condition in lepidopterous larvae, where there are a series of chambers separated by sphincters, to that in *Rhodnius* where the Malpighian tubules enter directly into the rectum (Wigglesworth, 1931b, 1932, 1953).

These examples serve to show that an attempt to generalize the part played by the individual organs of the excretory system in the regulation of salt and water balance in terrestrial insects will probably prove to be an oversimplification of the situation in any particular species. Nevertheless the picture which emerges from the detailed studies by Ramsay (1955a, b; 1956; 1958) on the function of the Malpighian tubules of *Dixippus* is not inconsistent, in principle, from that derived from less complete studies on the tubules of *Rhodnius* (Ramsay 1952), of some aquatic insects (Ramsay, 1950; 1951; 1953a, b), of the larvae of *Tenebrio* and *Pieris* (Ramsay, 1953b) and of *Schistocerca* (Phillips, 1961). Similarly, studies on the function of the rectum in a number of insects by Wigglesworth (1932), Ramsay (1955a) and Phillips (1961) suggest that this organ also has common properties.

a. The formation of the primary excretory fluid by the Malpighian tubules. The composition of the primary excretory fluid in a number of terrestrial insects is shown in Table XIII. In general the composition follows the same pattern as that of the aquatic insects (cf. Table II). The fluid is approximately iso-osmotic to the haemolymph, has less sodium (even when the sodium concentration of the haemolymph is very low, as in *Dixippus*) and has at least six times as much potassium. The chloride

concentration is about 20% below that of the haemolymph in the two insects—*Dixippus* and *Schistocerca*—in which it has been measured. The more complete analysis in *Dixippus* shows that the calcium and magnesium concentrations are also low, and that phosphate is the only ion, other than potassium, which is above the haemolymph level. In *Pieris*, *Tenebrio* and *Dixippus* the potential difference across the tubule wall is positive (with respect to the lumen), as it is in the aquatic insects, although in *Rhodnius* and *Locusta* it is reversed. In the last two species there is no obvious difference in the composition of either the tubule fluid or the haemolymph which can account for the reversal of polarity.

Calculation of the electro-chemical gradient for the potassium ion shows that the net potassium flux into the tubule lumen is always against the gradient, even in those species where the transtubular potential difference is negative (Ramsay, 1953b). The possibility of potassium movement due to solvent drag can be readily eliminated, as for the aquatic insects (see p. 332). There is no doubt that potassium is actively transported (Ramsay, 1953b).

In the case of sodium, in *Rhodnius* and *Locusta* the electro-chemical gradient favours the maintenance of the sodium flux by passive movement. But in the other species the gradient is very small, or in the wrong direction, although the evidence for active transport is not conclusive (Ramsay, 1953b). In the isolated tubules of *Dixippus*, however, movement of sodium can definitely take place against an electro-chemical gradient, and in a medium with a low potassium concentration the sodium concentrations of the tubule fluid rises above that of the haemolymph without a significant change in the potential difference (Ramsay, 1955b). Since sodium can be actively transported under certain conditions, it is highly likely that sodium movements always take place through a transport mechanism which may sometimes be working downhill.

In *Dixippus* it is probable that inorganic ions, other than sodium and potassium, enter the tubules passively. The relation between the concentration of these ions in the tubule fluid and their concentration in the medium surrounding the isolated tubules has been determined (Ramsay, 1956) and is shown in Fig. 10. Although the transtubular potential difference was not measured in these experiments, it is known that extensive changes in the composition of the medium have little effect on it (Ramsay, 1955b). Assuming a mean value of +25mV for the potential difference, lines of equal electro-chemical potential for monovalent and divalent cations and monovalent anions are also included in the figure. It can be seen that there is no significant evidence for the movement

TABLE XIII

The composition of the primary excretory fluid compared with that of the haemolymph in some terrestrial insects

Species	Concentrations in m equiv/l							Transtubular potential difference (mV)	Reference
	Osmotic pressure ≡ NaCl soln.	Na	K	Cl	Ca	Mg	PO_4		
Dixippus morosus									
Haemolymph	171	11	18	87	7	108	39	+21	Ramsay, 1953b, 1955a
Hindgut fluid*	171	5	145	65	2	18	51	+29	
Rhodnius prolixus									
Haemolymph	206	174	7	—	—	—	—		Ramsay, 1952, 1953b
Tubule fluid (distal part)	228	114	104	—	—	—	—	−35	
Schistocerca gregaria (water-fed animals)									
Haemolymph	214	108	11	115	—	—	—	—	Phillips, 1961
Hindgut fluid*	226	20	139	93	—	—	—		
Locusta migratoria									
Haemolymph	—	84	19	—	—	—	—	−16	Ramsay, 1953b
Tubule fluid	—	72	112	—	—	—	—		
Tenebrio molitor (larva)									
Haemolymph	—	65	42	—	—	—	—	+45	Ramsay, 1953b
Tubule fluid	—	15	250	—	—	—	—		
Pieris brassicae (larva)									
Haemolymph	—	7	23	—	—	—	—	+28	Ramsay, 1953b
Tubule fluid	—	5	167	—	—	—	—		

* This fluid was collected in the ligated hind-gut at the point of entry of the Malpighian tubules.

against the gradient of any of the ions shown, with the exception of sodium. Ramsay (1958) has shown that the kinetics of the excretion of organic substances, such as amino acids and sugars, by the tubules are consistent with the view that these substances enter the tubule lumen by passive diffusion through its wall. In view of the apparent absence of saturation or mutual interference effects, specific transport systems do not seem to be involved. It is therefore possible that those inorganic ions, other than potassium and sodium, which move through the tubule wall

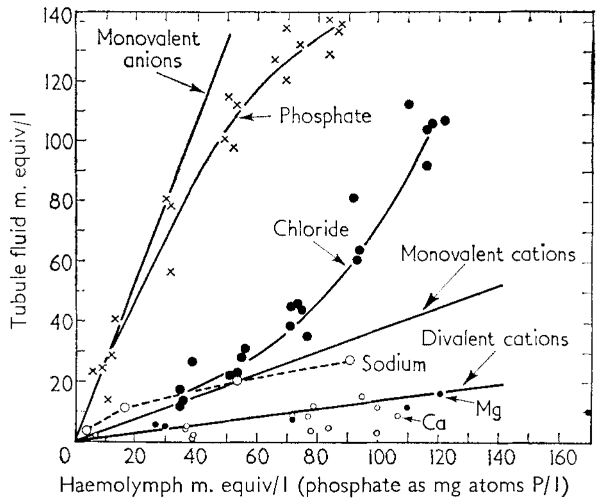

FIG. 10. The relationship between the concentration of phosphate, chloride, sodium, calcium and magnesium in the fluid secreted by the isolated Malpighian tubules of *Dixippus*, and their respective concentrations in the haemolymph. The straight lines represent lines of equal electro-chemical potential for mono- and divalent cations and monovalent anions for a transtubular P.D. of +25 m.v. If the concentration of an ion in the tubule fluid is above the appropriate equi-potential line, then this is evidence for the movement of the ion against an electro-chemical potential gradient.

References: Ramsay, 1955b, 1956.

in the direction determined by the electro-chemical gradient, do so by passive diffusion as well.

Caution is necessary, however, in extending these conclusions to the tubules of species other than *Dixippus*, since detailed analyses of the tubule fluid are not yet available in other forms. One possible difference can already be anticipated in the case of chloride in *Schistocerca* (see Table XIII). The observed tubule fluid/haemolymph chloride ratio could only be maintained passively by a potential difference more positive than about −5 mV, yet in the related species, *Locusta migratoria*, the

observed potential difference is lower (−16 mV). If a negative potential difference of this size is also found in *Schistocerca* then active transport of chloride into the tubule would be required. It is possible that active transport of anions may be found in other species and this could account for the negative potentials found in some forms.

Despite the possibility of the active transport of ions other than potassium, there is no doubt that the secretion of potassium has a rôle of special importance in the formation of the Malpighian tubule fluid.

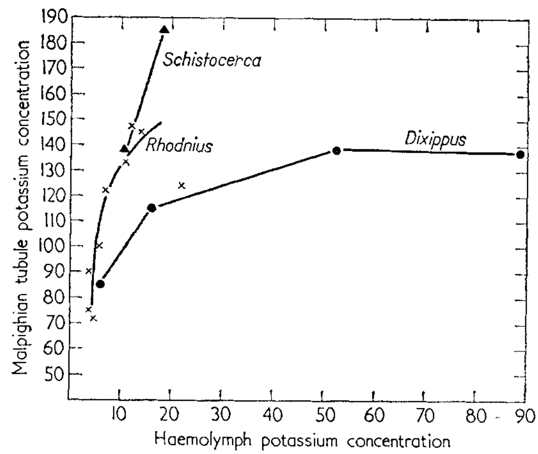

FIG. 11. The relationship between the potassium concentration (mM/l) of the haemolymph and that of the Malpighian tubule fluid (mM/l) (distal region of the tubule, in the case of *Rhodnius*).

References: *Dixippus*—Ramsay, 1955b; *Rhodnius*—Ramsay, 1952, 1953b; *Schistocerca*—Phillips, 1961.

There are several features in the behaviour of potassium which deserve particular note. Firstly, in all the tubules examined the potassium concentration in the fluid is exceptionally high. Secondly, small increases in the haemolymph potassium concentration result in substantial increases in that of the tubule fluid. This is seen in *Rhodnius*, in the upper tubule fluid during the diuresis period after feeding on a blood meal containing added KCl (Ramsay, 1952); in the isolated tubules of *Dixippus* (Ramsay 1955b), and in *Schistocerca* after raising the haemolymph potassium by giving fasting animals saline, containing KCl, to drink (Phillips, 1961, and Table XVIII). The relation between the haemolymph and tubule fluid potassium concentration is shown in Fig. 11. It may be noted that in *Dixippus* the tubular concentration reaches a maximum when the haemolymph concentration exceeds about 50 mM/l—a fact which may

be due simply to the limitation imposed by the osmotic pressure of the tubule fluid not exceeding that of the haemolymph.

The third, and perhaps the most important feature of potassium, is revealed by studies on the rate of fluid production in the isolated tubules of *Dixippus* (Ramsay, 1955b). The potassium concentration of the haemolymph is an extremely important factor in determining the rate of flow—the two bearing a direct, and approximately linear relation to each other.

The high rate of potassium secretion by the tubules (and its subsequent reabsorption in the hind-gut), together with the sensitivity of the flow rate to the haemolymph potassium concentration, provide the basis for the theory advanced by Ramsay (1953b, 1954, 1955b, 1956) that the active transport of potassium is fundamental to the production of the tubule fluid, and is the prime mover in generating the flow of urine. Careful measurement of the osmotic pressure of the tubule fluid in *Dixippus* showed that the fluid formation could not be due to passive water movement as a result of the establishment of an osmotic gradient by the inward transport of potassium across the tubule wall. In some cases, in fact, water movement took place against a slight gradient (Ramsay, 1954). However, a driving force for water movement can also be provided by frictional interaction between water and other moving substances (see Section II; and Ramsay, 1956, for the possibility of electro-osmosis). Diamond (1962) has shown that the interaction between an actively-transported substance (NaCl) and water can be strong enough to carry water against a large osmotic gradient, and there is no reason to suppose that the active transport of potassium could not equally well do the same. Although the possibility of such a mechanism operating in the tubule would require rigorous testing, it is strongly supported by the fact that water movement into the tubule lumen is closely geared to potassium uptake. Discussion on the precise mechanism involved in tubule fluid formation cannot be taken further at present; but it is clear that Ramsay's theory that the active transport of potassium generates the flow of tubule fluid is readily acceptable, and the fact that a slight adverse osmotic gradient may be present does not make it less likely to be true.

It is possible that sodium movement may also contribute towards tubule fluid production under certain circumstances. In the isolated tubules of *Dixippus* the rate of tubule fluid secretion is increased somewhat when the concentration of sodium in the medium is very high (Ramsay, 1955b). In view of the fact that sodium also appears to be moved by a transport system, it is possible that some water is carried

simultaneously in the same manner as envisaged for potassium. However, at the normal sodium concentration of the haemolymph the amount of water moved would be negligible compared with that moved by potassium transport.

One of the surprising features of the isolated Malpighian tubules of *Dixippus* is that they function normally in haemolymph with its normal ionic composition grossly changed, yet are unable to survive in any known artificial medium (Ramsay, 1956). There are, however, some limitations to the changes in haemolymph concentration which can be made. The rate of secretion is normal if the haemolymph pH is kept within the range 5·7–7·3, but falls markedly beyond these limits. Of the other ions studied (excluding sodium and potassium) the only serious reduction in the rate of flow is caused by an increase in the calcium concentration above about 90 m equiv/l (Ramsay, 1956).

In normal animals it is highly likely that the rate of fluid secretion is controlled by factors other than the direct effect of the concentration of a particular ion, such as potassium, in the haemolymph. In *Rhodnius*, Wigglesworth (1931a) found that urine production is very rapid for the first few hours following a blood meal. Recently Maddrell (1962) has shown that the diuresis is due to the action on the tubules of a hormone released into the haemolymph from neurosecretory cells in the ganglionic mass of the mesothorax.

b. Regional differentiation of function in the Malpighian tubules. In *Rhodnius*, each of the four Malpighian tubules is divided into two regions with an abrupt transition separating them. The distal part is translucent and practically colourless with cells having a striated border of closely packed but discrete elements. The proximal part appears white and opaque—the cells also have a striated border but the filaments are far less regular in size and arrangement (Wigglesworth, 1931b; Wigglesworth and Salpeter, 1962). Dyes which are secreted by the tubules are taken up rapidly in the distal part and transferred to the lumen. Ligaturing also shows that the distal part is secreting fluid, whereas in the proximal region no secretion takes place and probably fluid is reabsorbed (Wigglesworth, 1931c). Measurement of the composition of the fluid in the two regions gives further evidence of the difference in function—see Table XIV. In the distal part of the tubule the fluid has the characteristic pattern of a high potassium concentration and a sodium concentration below that of the haemolymph: the osmotic pressure is significantly above that of the haemolymph. The differences in sodium and potassium concentrations are accentuated in animals fed with blood mixed with potassium chloride. The differences in concentration

established by the activity of the distal part of the tubule are degraded in the proximal portion. The osmotic pressure falls to that of the haemolymph, but since Wigglesworth (1931c) has shown that water is not added (but is probably absorbed) in this region of the tubule, the changes in concentration cannot be explained solely in terms of a passive equilibration (Ramsay, 1952). Potassium is certainly reabsorbed, but a decision concerning the direction of sodium movement must await a quantitative determination of the extent of water reabsorption.

TABLE XIV

A comparison between the composition of the haemolymph and of the fluid in the distal and proximal parts of the Malpighian tubules of *Rhodnius* (from Ramsay, 1952)

Fluid	Concentrations in mM/l			Concentrations in mM/l		
	Composition 24—29 h after a normal blood meal			Composition $\frac{1}{2}$–$1\frac{1}{4}$ h after a meal of blood with added KCl		
	Na	K	Osmotic pressure ≡ NaCl soln	Na	K	Osmotic pressure ≡ NaCl soln
Haemolymph	174	7	206	150	20	181
Distal (upper) part of tubule	114	104	228	79	144	224
Proximal (lower) part of tubule	158	50	199	92	105	182

In *Dixippus*, also, some differentiation in physiological properties along the length of the tubules is clearly established (Ramsay, 1955b). The superior tubules show some slight gradation in appearance: the inferior tubules are similar but, in addition, their distal ends are dilated and packed with white granules consisting mainly of calcium carbonate. Collection and analysis of fluid produced by the superior tubule divided into three approximately equal lengths provides the evidence for differentiation in function of the different regions (Ramsay, 1955b). The potassium/sodium ratio of the fluid secreted by the distal portion is greater than that from the more proximal regions. In this respect it is similar to the tubules of *Rhodnius*, but it differs in that all parts of the tubule are secretory—the observed differences being due to the relative rates of secretion of sodium and potassium. It may be noted that even in the proximal part of the tubule potassium is secreted about ten times

more rapidly than sodium. The inferior tubules appear to be similar in function, except for the distal dilation—here no secretion takes place and there is some transfer of potassium from the lumen to the medium, although this is very small compared with that passing down the tubule to the gut (Ramsay, 1955b).

c. Ionic regulation by the Malpighian tubules. In the isolated tubules of *Dixippus* it is possible to calculate the total quantity of an ion

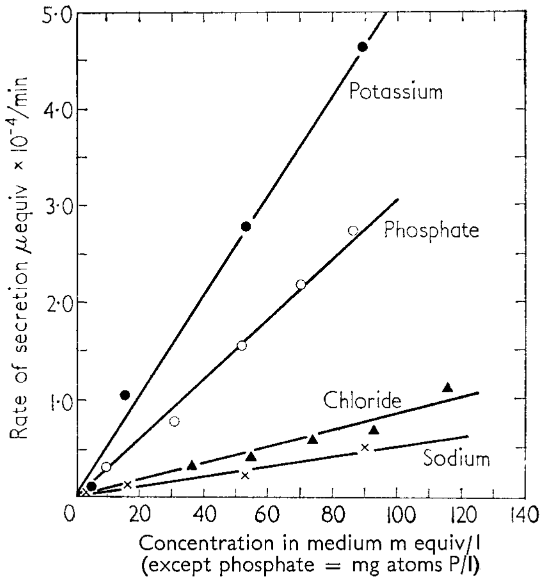

FIG. 12. The relationship between the rate of secretion of inorganic ions by the isolated tubules of *Dixippus* and their respective concentrations in the haemolymph.
References: Ramsay, 1955b, 1956.

secreted in a given time (the rate of secretion) from a knowledge of the rate of flow of the tubule fluid and from its ionic composition, and to relate this to concentration of the ion in the bathing medium. Calculation of the rates of secretion of sodium and potassium showed that they are approximately proportional to the concentration of the ions in the haemolymph (Ramsay, 1955b). A similar conclusion can be drawn from calculation of the rates of secretion of phosphate and chloride from the data given by Ramsay (1956). These results are illustrated in Fig. 12. But, as Ramsay (1956) has stressed, there is nothing in the response of the tubules to variations in the haemolymph concentrations which might suggest that the tubules themselves are responsible for the maintenance

of the normal composition of the haemolymph. Since the composition of the tubule fluid is markedly different from that of the haemolymph under all conditions, its secretion must always have the effect of altering the haemolymph composition. There is no known case where the concentration of an ion in the tubule fluid is below that in the haemolymph at low haemolymph concentrations, but rises above it when the concentration is increased—a requirement for regulation to be effected.

These considerations, however, do not deny the Malpighian tubules a positive rôle in the overall process of regulation. The function of the tubules is clear: they generate a flow of fluid which presents a variable load to the reabsorbing system. The quantity of any ion (or of water) which is finally discharged from the rectum depends on the difference between the rate at which it is secreted and its rate of reabsorption.

d. The rôle of the hind-gut. The part played by the hind-gut (not including the rectum) in modifying the primary excretory fluid is still largely unknown. Visual observations by Wigglesworth (1932) suggest that in many insects some water reabsorption takes place in the hind-gut before the rectum is reached. Ramsay (1955a) found in *Dixippus* that the composition of the intestinal fluid in fasting animals was not significantly different from that of the fluid emerging from the Malpighian tubules. Injection of the tubule fluid into the ligated intestine, however, showed that a slow absorption took place, although the composition of the fluid was not changed. There was some indication of a slight change in composition of the intestinal fluid in animals injected with sodium chloride (Ramsay, 1955a).

e. The rôle of the rectum. There is no doubt that the rectum plays a major rôle in the modification of the primary excretory fluid and that the process of regulation is largely effected here. Wigglesworth (1932) clearly demonstrated that in many insects which produce relatively dry faeces the rectum is the main site of water reabsorption. Patton and Craig (1939) found that radioactive sodium passing down the Malpighian tubules of the larva of *Tenebrio* passed through the rectal wall, showing that the rectum is permeable to sodium. Wigglesworth (1931a) found that in *Rhodnius*, after the diuresis period following a blood meal, the rectal fluid osmotic pressure rises steadily until after 48 h it becomes approximately twice that of the haemolymph. This was confirmed by Ramsay (1952) who also showed that the potassium concentration of the rectal fluid, which is low at first, increases in parallel with the osmotic pressure, whereas the sodium concentration (which is slightly below the haemolymph level) increases only a little. In assessing the part played by the rectum—and in *Rhodnius* this must include the

Malpighian tubule ampullae from which cells project into the rectum—one must compare the composition of the fluid from the proximal parts of the tubule (as representing the fluid entering the rectum) with that of the rectal fluid. This is possible for the period 19–29 h after normal feeding for which both measurements are available (Ramsay, 1952, and Table XV).

It is clear that the observed changes could be due to potassium secretion into the rectum, and this is not ruled out. However, as the fluid contains much sediment at this stage it is much more likely that water is reabsorbed against an increasing osmotic gradient. If this is so,

TABLE XV

The composition of the haemolymph, the fluid from the proximal part of the Malpighian tubules, and the rectal fluid, in *Rhodnius*, 19–29 h after a normal blood meal (from Ramsay, 1952)

Fluid	Concentrations in mM/l		
	Na	K	Osmotic pressure ≡ NaCl soln
Haemolymph	174	7	206
Proximal (lower) part of tubule	158	50	199
Rectal fluid	161	191	358

then sodium must also be reabsorbed in proportion, since its concentration remains relatively unchanged.

In *Dixippus* measurement of the rate of loss of sodium and potassium in the faeces of fasting animals, compared with the calculated secretion of these ions into the hind-gut by the Malpighian tubules, showed that 95% of the sodium and 80% of the potassium is reabsorbed. Fluid squeezed from the rectum had an osmotic pressure, in some cases, of over three times that of the haemolymph and contained very much more potassium than sodium (Ramsay, 1955a). The rectum is therefore the site of reabsorption of water against an osmotic pressure gradient and also of the reabsorption of sodium and potassium.

Further evidence for the view that the rectum of terrestrial insects is a region where reabsorption of water and salts takes place, comes from studies on the mechanism of absorption in the rectum of *Schistocerca* and *Calliphora* (Phillips, 1961).

f. The mechanism of water absorption in the rectum. In the locust rectum the process of water absorption has been followed by injecting experimental solutions into the rectum after ligaturing at its junction with the hind-gut (Phillips, 1961). Water reabsorption was calculated from the volume change of the injected solution using a non-absorbed substance, such as ^{131}I-albumin, as an indicator. In fasting animals given tap water to drink, only 31 % of a solution of xylose hyperosmotic to the haemolymph (freezing point depression, $\Delta = 1{\cdot}08$°C, compared with a Δ of 0·75°C for the haemolymph) introduced into the rectum remained after 5–9 h. During the absorption of water the osmotic pressure of the rectal contents increased to $\Delta = 1{\cdot}74$°C. It follows, therefore, that in the locust rectum a net uptake of water can take place against an osmotic gradient and that the gradient is increased during the reabsorption. In these experiments some of the xylose is also absorbed, but the uptake of water is not associated with simultaneous solute uptake since the same result is obtained if a non-penetrating solute, such as trehalose, is used (Phillips, 1961).

Measurement of the hydrostatic pressure in the rectum during absorption gave a maximum value of only 12 cm H_2O—a negligible pressure compared with the osmotic pressure against which the water absorption can take place. Phillips (1961) also considered the possibility that the water uptake might be due to electro-osmosis, since a small potential difference (15–32 mV lumen positive to the haemolymph) across the rectal wall was observed. This proved to be an unimportant factor, since after the reversal of the potential by passing a current of 10 μA across the wall, water was still absorbed against an osmotic gradient and the osmotic pressure of the rectal fluid increased. Similarly the passage of currents of 10 μA and 50 μA in the opposite direction did not bring about a significant change in the normal rate of water absorption.

During all the water uptake experiments with xylose or trehalose solutions, the total ionic concentration in the rectum remained very low (about 5 m equiv/l) and the simultaneous net uptake of ions was extremely small.

These experiments establish that the absorption of water in the rectum takes place (*a*) against an osmotic gradient and in the absence of a significant hydrostatic pressure difference, (*b*) in the absence of significant active or passive solute movement, (*c*) at a rate which cannot be accounted for by electro-osmosis, and (*d*) with an increase in the osmotic pressure of the rectal fluid during absorption. They provide very strong evidence in favour of the view that the movement of water across the rectal wall can only be explained by the presence of an active transport

mechanism for water, which is independent of other mechanisms for transporting solutes (Phillips, 1961, and see Section II).

The rate of water uptake in the rectum depends on the osmotic gradient across the wall. There is a maximum gradient against which water can be absorbed and if this is exceeded then water moves into the rectum. Figure 13 shows the initial uptake rate in the rectum of fasting locusts supplied with tap water, in relation to the osmotic pressure of the rectal contents. In the absence of an osmotic gradient water is taken

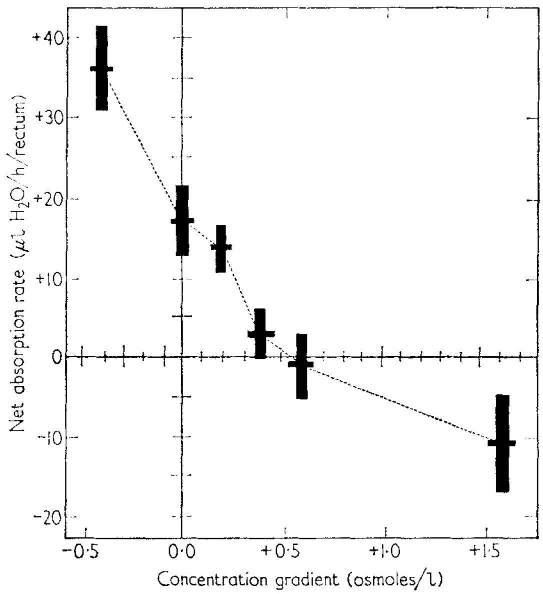

FIG. 13. The relationship between the initial osmotic pressure gradient (expressed in OSM/l) and the net rate of water movement across the rectal wall. A positive sign indicates movement from the lumen (ordinate) or rectal fluid more concentrated than haemolymph (abscissa). From Phillips, 1961.

up at about 17 μl/h, but is reduced to zero when a gradient of 0·6 osmole is present. The relation between the uptake rate and the osmotic gradient is approximately linear except, apparently, at the higher rectal concentrations. Over the linear region it is likely that the active uptake component (17 μl/h) is superimposed on a passive component due to the diffusion of water in the direction of the osmotic gradient (Phillips, 1961).

g. The regulation of water absorption in the rectum. The osmotic gradient against which water uptake can take place is determined by the

water requirements of the animal. This has been demonstrated by comparing the maximum osmotic pressure attained in the rectal fluid of fasting locusts supplied either with tap water, or with a hyperosmotic saline solution (containing 300 mM/l NaCl and 150 mM/l KCl) to drink. A 0·9 M trehalose solution was introduced into the ligated rectum in each case and the osmotic pressure of the rectal contents measured after 3 h (Phillips, 1961—Table XVIa). The osmotic pressure of the rectal fluid of the saline-fed locusts is over twice that of the water-fed animals and the osmotic gradient about three times as great. The animals can clearly regulate the degree of concentration in the rectum; but it is not

TABLE XVI

The osmotic pressure of the haemolymph and rectal fluid 3–5 h after the injection of either (*a*) 0·9 moles trehalose, or (*b*) a saline–trehalose solution, into the ligated recta of water-fed and saline-fed locusts, and the maximum osmotic pressure gradient developed across the rectal wall (from Phillips, 1961)

Experimental conditions	Osmotic pressure as freezing point depression (°C)		
	Haemolymph	Rectal Fluid	Osmotic gradient
a. 0·9 moles trehalose			
(i) Water-fed animals	0·74	1·30	0·56
(ii) Saline-fed animals	0·96	2·74	1·78
b. Saline–trehalose (containing 300 mM/l NaCl and 50 mM/l KCl)			
(i) Water-fed animals	0·72	1·53	0·81
(ii) Saline-fed animals	0·92	2·91	1·99

known whether this is brought about by an increase in the active uptake rate or to a reduction of the permeability of the rectal wall to passive water movement.

The normal rectum contains a high concentration of salts which are simultaneously reabsorbed with water—but this does not appear to enhance the rate of water uptake. This has been shown by injecting into the ligated rectum solutions containing trehalose with sodium chloride added to a concentration of 300 mM/l. The degree of concentration of the rectal contents in both water-fed and saline-fed locusts is not significantly different from that found for trehalose solutions alone (Phillips, 1961—Table XVIb). The fact that the rate of water absorption is not increased by simultaneous salt uptake is also shown by measurement of water uptake from a locust Ringer solution introduced into the rectum of water-fed animals. Under these conditions a large volume

decrease occurs due to the uptake of water and salts, but the initial rate of water uptake (20 μl/h) is only slightly greater than that found for xylose solutions (Phillips, 1961).

Active water uptake and control of the degree of concentration within the rectum also appear to be characteristic features of the rectum of *Calliphora* (Phillips, 1961).

h. The mechanism of ion absorption in the rectum. Active transport mechanisms are also involved in the uptake of inorganic ions in the locust rectum. This has been demonstrated by a study of the uptake of ions in the ligated rectum of water-fed locusts (Phillips, 1961). Ion absorption was followed after the injection into the rectum of a Ringer solution containing sodium, potassium and chloride in approximately the same concentrations as in the haemolymph, but made hyperosmotic by the addition of xylose in order to prevent simultaneous water absorption. After 3–6 h a large percentage of all the ions had been absorbed (31 % of the sodium, 44 % of the chloride and 75 % of the potassium) and the concentrations of the three ions reduced below their respective concentrations in the haemolymph. Thus in each case absorption takes place against a concentration gradient. Further, since in most of the experiments water moved into the rectum, it is clear that ion absorption is not dependent on simultaneous water uptake. The rate of chloride uptake (0·2 μM/h) was approximately equal to the sum of the sodium uptake rate (0·16 μM/h) and that of potassium (0·06 μM/h).

In the normal rectum the potential difference across the rectal wall is 19 mV (lumen positive to haemolymph). In the ligated rectum, after the introduction of the Ringer-xylose solution the potential difference rises to 35 mV, but declines to the normal value after some hours. Chloride uptake, therefore, takes place against the electro-chemical gradient and in the absence of water uptake, hence the presence of an active transport mechanism for chloride is established (Phillips, 1961).

Active transport of sodium and potassium also occurs, and is in evidence when the rectal concentrations are low. This has been demonstrated by the injection into the ligated rectum of a saline-xylose of the same osmotic pressure as before but of low ionic concentration (Na = 25 mM/l; K = 5 mM/l). After equilibrium was reached (2–3 h) the concentrations of ions in the rectum had been reduced. For the maximum concentration gradients recorded, the potential difference necessary for passive movement was calculated and compared with the observed value—Table XVII. It is clear that active transport of both sodium and potassium must take place in order to reduce the concentrations in the rectum to the low values observed (Phillips, 1961).

Thus during the reabsorption of a Ringer solution from the rectum, chloride is transported actively from the start; sodium and potassium may be taken up largely passively at first, but as their concentration in the rectum falls, active transport mechanisms must take over.

i. The regulation of ion absorption from the rectum. The rate of uptake of inorganic ions in the locust rectum is regulated in relation to the ionic concentration of the haemolymph. The rate of uptake of the three ions, sodium, potassium and chloride, has been studied at different rectal concentrations by introducing into the ligated rectum xylose solutions containing concentrations of sodium chloride in the range 20–650 mM/l,

TABLE XVII

The minimum concentrations of inorganic ions in the ligated recta of water-fed locusts (from Phillips, 1961)

Ion	Haemolymph concentration C_H (mM/l)	Rectal fluid concentration C_R (mM/l)	$\frac{C_R}{C_H}$	Calculated potential difference across rectal wall for passive movement (mV)	Observed potential difference (mV)
Chloride	115	4	0·05	—	—
Sodium	106	9·6	0·09	+66	+20
Potassium	9	0·2	0·02	+94	+22

and with potassium chloride added for the potassium uptake measurements (Phillips, 1961). In water-fed locusts the rates of uptake of sodium and chloride are approximately proportional to the concentrations in the rectal fluid. Figure 14 shows the relationship found for chloride—that for sodium is essentially the same. Potassium also behaves in a similar manner (Fig. 15) but it is absorbed about ten times as rapidly as sodium and chloride at the same concentration. From the more concentrated solutions the gradient favours a passive uptake of the ions and it is probable that the measured uptake rate is due both to active transport (which is probably rate limited at the higher concentrations) and to passive uptake; the latter becoming more important as the rectal concentration is increased.

In saline-fed locusts, in which the haemolymph sodium and chloride is some 40% and the potassium some 70% higher than in the water-fed

animals, the relation between the uptake rate and rectal concentration for all the ions is of a different character (Figs. 14 and 15). At the lower rectal concentrations the difference is not marked—and this is confirmed by the fact that the maximum ionic gradients which can be maintained in the rectum are also not very different from those in the water-fed animals—but at higher rectal concentrations it is apparent that a maximum rate of absorption is attained. As a consequence of this, the

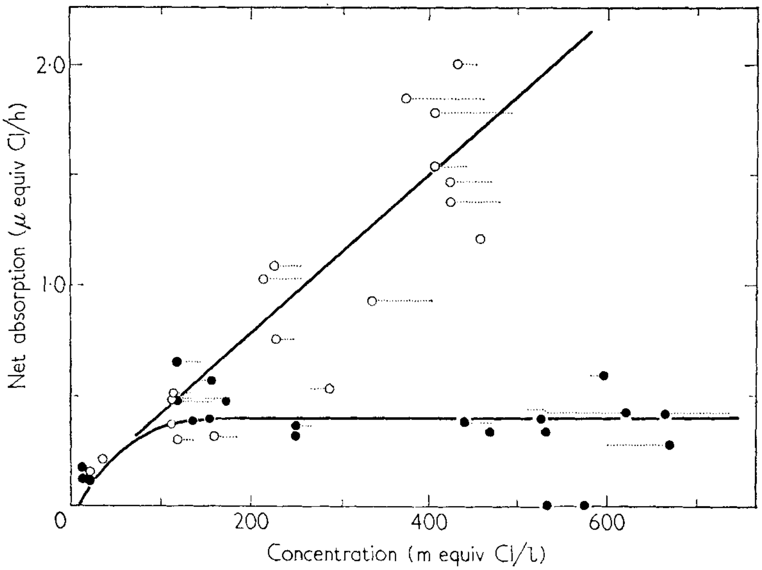

FIG. 14. The relationship between the mean rectal fluid concentration and the net rate of absorption of chloride from ligated recta of water-fed locusts (open circles) and saline-fed locusts (solid circles). Broken horizontal lines extend from the mean to the initial concentration. From Phillips, 1961.

difference in the rate of ion uptake in the recta of saline-fed locusts as compared with water-fed ones, increases markedly as the rectal fluid concentration is increased.

It is possible, at least in the case of chloride, that the difference between the water-fed and the saline-fed condition is not due to a reduction in the rate of active transport of the ions but to a change in the passive permeability of the rectal wall: this would explain the fact that the difference is most marked at high rectal concentrations (Phillips, 1961).

j. Ionic regulation by the excretory system as a whole. It has already been seen that the quantity of any substance removed by the excretory

system depends on the difference between its rate of secretion and the rate of its subsequent reabsorption. This may now be illustrated by two examples. In *Dixippus* the reabsorption of sodium is normally highly efficient, and during starvation for 96 h the haemolymph sodium concentration is virtually unchanged. Injection of sodium chloride, which raised the haemolymph concentration to 4–5 times the normal value, was followed by enhanced excretion. The rate of secretion by the Malpighian tubules was increased 3–4 times and only about 40% of the sodium was reabsorbed. Nevertheless the regulation is not particularly

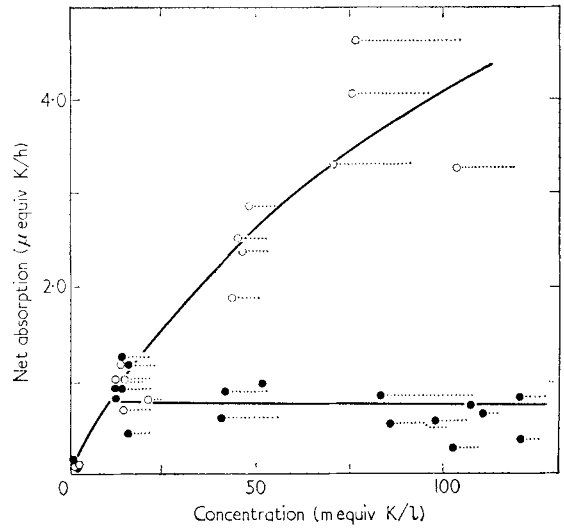

FIG. 15. The relationship between the mean rectal fluid concentration and the net rate of absorption of potassium from ligated recta of water-fed locusts (open circles) and saline-fed locusts (solid circles). Broken horizontal lines extend from the mean to the initial concentration. From Phillips, 1961.

impressive, as in 3 days only about a third of the injected sodium had been removed (Ramsay, 1955a). For the second example we return to the locust *Schistocera gregaria*, and compare, in Table XVIII, the Malpighian tubule secretion with that of the rectal fluid in fasting animals supplied with either water or saline to drink (Phillips, 1961). In the water-fed animals the rate of reabsorption of ions in the rectum more than keeps pace with their rate of supply from the hind-gut fluid and low rectal concentrations result—just as in the case of solutions introduced into the ligated rectum—but, at the same time, only very small amounts of rectal fluid are produced. This is clearly due to water

reabsorption also keeping up with the supply from the hind-gut, and fasting water-fed animals are thus unable to excrete excess water; although they can retain salts very effectively. Water can be excreted in feeding animals, however, as unabsorbed organic solutes from the food pass into the rectum and serve to retain large amounts of water there, while salts are actively absorbed.

TABLE XVIII

The composition of the haemolymph, hindgut fluid and rectal fluid of water-fed and saline-fed locusts (from Phillips, 1961)

	Concentrations in mM/l			Freezing point depression °C	Collection rate µl/h
	Cl	Na	K		
Water-fed locusts					
Haemolymph	115	108	11	0·74	—
Hind-gut fluid	93	20	139	0·78	8
Rectal fluid	5	1	22	1·52	—
Saline-fed locusts					
Haemolymph	163	158	19	0·96	—
Hind-gut fluid	192	67	186	—	7
Rectal fluid	569	405	241	3·47	—

In the fasting, saline-fed animals, on the other hand, a large volume of rectal fluid collects and becomes highly concentrated (see Table XVIII), the osmotic pressure being slightly higher than that produced in the ligated rectum of saline-fed animals. Hind-gut fluid is produced at much the same rate and is rather more concentrated than in the water-fed animals, but, due to the curtailed reabsorption of ions in the rectum, the rectal fluid osmotic pressure rises sharply as water is absorbed. As the osmotic gradient increases the rate of water absorption also slows down: the net result is that neither water nor salt reabsorption keep pace with their rate of entry from the hind-gut and a highly concentrated fluid gradually accumulates. The overall effect is to reduce the haemolymph concentration.

C. THE WATER RELATIONS OF TERRESTRIAL INSECTS

Various aspects of the water relations of terrestrial insects have been the subject of several recent reviews (Edney, 1957; Richards, 1951, 1958; Wigglesworth, 1957; Beament, 1954, 1961a), and treatment here

is therefore restricted to a brief summary in relation to the overall maintenance of water balance in the terrestrial forms.

The insects (and some other Arthropoda) are the only very small animals which have become adapted to life in dry habitats and, in view of their small size, the conservation of water must be of great importance to the majority of the terrestrial species. Water may be lost through the excretory system and through the respiratory surfaces and integument. The excretory loss is generally minimized by the production of relatively dry faecal pellets, the main nitrogenous component being uric acid. Loss through the respiratory surface is small as they are largely internal, and is further reduced by the closure of the spiracles (Mellanby, 1934; Wigglesworth, 1935; Wigglesworth and Gillet, 1936). In addition, the cuticle of most terrestrial species is very impermeable to water, a property conferred on it by the presence in the epicuticle of one (or sometimes two) exceedingly impermeable organized monolayers of polar wax molecules (Beament, 1958, 1959). It has now been conclusively demonstrated by an elegant technique (Beament, 1958, 1959) that in most insects the cuticular permeability is greatly increased at certain characteristic transition temperatures. This is attributed to the thermal disorientation of the wax monolayer. Ecological temperatures are usually well below the transition temperature (but see Beament, 1961b). Beament (1958, 1959) measured the transpiration through the cuticle (including spiracles) in dry air for a number of recently-killed terrestrial insects, the apparatus being designed to prevent the build-up of humidity gradients around the specimens. Some of these results are shown in Table XIX, and have also been recalculated and expressed as mgm of water lost per animal per h in air of 60% relative humidity (R.H.) and 20°C (extrapolating slightly to this temperature, where necessary). Even in the most permeable forms the loss rate accounts for a very small percentage of the total body weight per h.

Govaerts and Leclercq (1946) studied the exchange of water between some insects and the water vapour of a saturated atmosphere, using D_2O. They found that the period of exchange with the atmosphere varied between 5 and 13 days in different insects, although they did not attempt to calculate the D_2O fluxes. However, the assumption of an average figure of 9 days for the exchange to be 93% complete would lead to an influx equivalent to about 1·2% of the total water per h. If the water fluxes are proportional to the water activities inside and outside the insect, then the water efflux will be of the same order as the influx (since the activities are not very different). In theory this might be expected to be the same as the net loss of water into completely dry air

TABLE XIX

The transpiration of water through the cuticle of some terrestrial insects (data from Beament, 1958, 1959)

Species	Temperature °C	Approx.‡ Weight mgm	mgm water lost /animal/ mm*Hg/h	Recalculated as mgm water lost /animal/h at 20°C and 60% R.H.	As % of initial weight per h	Recalculated as mgm water lost /animal/h at 20°C and 0% R.H.	As % of initial water per h‖
Periplaneta nymph	25·4	800§	0·113	1·03†	0·13	3·0†	0·5
Nematus larva	20	80	0·05	0·316	0·4	0·850	1·5
Pieris larva	20	370	0·107	0·676	0·18	1·82	0·70
Pieris Pupa	20	370	0·012	0·076	0·02	0·204	0.08
Rhodnius nymph	20	100	0·0016	0·010	0·01	0·027	0.04
Tenebrio larva	20	90	0·071	0·449	0·50	1·21	1·9
Tenebrio pupa	20	90	0·001	0·006	0·007	0·017	0·03

* Saturation deficiency expressed as mmHg.
† Calculated for 25°C and R.H. of 60% or 0%.
‡ From Wigglesworth, 1945.
§ Probable weight (approx. 80% of that of adult).
‖ Initial water assumed to be 70% of the weight.

and it is interesting to see if this is so. The last column of Table XIX shows Beament's results calculated as a percentage of the initial water lost per h in completely dry air at 20°C, and it can be seen that for *Periplaneta*, *Nematus*, *Pieris* and *Tenebrio* larvae a figure of the order of 1 % of the body water per h is also found. In their experiments with D_2O Govaerts and Leclercq did not include the more impermeable forms, such as *Rhodnius* nymphs nor the pupae of *Tenebrio* and *Pieris*, but in *Tenebrio* larvae the exchange was complete in 13 days. Their results, therefore, show a general agreement with those obtained by the transpiration method, and suggest that heavy water may prove a useful additional tool for the study of certain aspects of the water relations of insects.

It is difficult to assess to what extent the water loss recorded in laboratory experiments represents the losses which actually take place under field conditions. Respiratory movement of air through the tracheae and, also, possibly damage to the cuticle would tend to increase the loss rate. On the other hand closure of the spiracles, the occupation of favourable micro-environments and the build-up of humidity gradients around the insect would tend to reduce it. However, it is safe to conclude that, in general, the water loss is small and is probably of the same order of magnitude as that determined by transpiration experiments (Table XIX). This view receives some support from the results of Wigglesworth and Gillett (1936); they found that a living *Rhodnius* nymph (not excreting) kept at 24°C in dry air over sulphuric acid lost water at a rate equal to 0·12% of its initial weight per h. Assuming a R.H. of 10% in the air (see Beament, 1958) this corresponds to a loss of 0·04% at 60% R.H. and 20°C, and may be compared with the value of 0·01% from Table XIX.

If an insect is to remain in water balance, water lost by transpiration and excretion must be replaced by water from the environment, although in the case of pupae in dry habitats it seems likely that the insect relies completely on the impermeability of the cuticle for the retention of sufficient water; the same also applies to various insect eggs. Many insects drink water (Leclercq, 1946), but it appears that drinking is confined to adult forms and that the larvae rely on their diet for water. Water may also be gained by (*a*) the retention of metabolic water, in forms living on dry diets (Edney, 1957; Wigglesworth, 1953); (*b*) absorption through the cuticle (Ramsay, 1935; Wigglesworth, 1953); and (*c*) the absorption of water vapour—found in a few species at certain stages of their life histories (*Tenebrio molitor* larvae, Buxton, 1930; Mellanby, 1932; *Chortophaga viridifasciata* nymphs, Lugwig, 1937; prepupae of

the rat flea *Xenopsylla brasiliensis*, Edney, 1947). This last process, which has been discussed recently by Edney (1957) and Beament (1961a), is of great interest. It appears to be a special case of active water transport since water may be absorbed against a very steep osmotic gradient, of as much as 5·5 osmoles (or more in *Xenopsylla*)—this may be compared with the maximum osmotic gradient of just over 1 osmole against which water can be moved in the rectum of saline-fed locusts (Phillips, 1961). The site of the water absorption in insects is not known at present, but in ticks which also have the ability to absorb water vapour it appears that the uptake occurs through the cuticle (Lees, 1946; Browning, 1954). The absorptive mechanism, which is at present quite obscure, appears to respond to relative humidity rather than saturation deficiency (Mellanby, 1932) and so its control is likely to be complex. The ecological importance of this type of absorption is not clear, but as it generally takes place from high relative humidities (70% or greater) it seems possible that its value may lie in rendering the insect independent of a supply of *liquid* water.

Some insects can tolerate a considerable reduction in the volume of their haemolymph, which appears to function as a store of water which can be drawn upon in times of dessication (Mellanby, 1939). In *Locusta* and *Schistocerca* kept in dry conditions the haemolymph volume may be reduced so much as to make collection impossible (Hoyle, 1954; Phillips, 1961). In the case of *Schistocerca* there is a rise in osmotic pressure in the reduced haemolymph, but it is very much less than would be expected on the basis of the reduction in volume.

VI. Regulation of the Ionic Composition of the Tissues

In general the tissues of insects show a high concentration of potassium and a low concentration of sodium (Tobias, 1948a, b; Ramsay, 1953a; Shaw, 1955b). Some insects, mainly Lepidoptera and Hymenoptera, have a high concentration of potassium and a low concentration of sodium in the haemolymph (see Table X), and on the basis of results from other organisms (Hodgkin, 1951) their nerves might be expected to be largely depolarized. Although a study of nerve function in such insects would be of great interest, it has not yet been attempted. Many insect tissues, including muscle, are surrounded by a sheath of connective tissue (Wigglesworth, 1953, 1956). In the case of the central nervous system the sheath consists of an outer fibrous layer of connective tissue (neural lamella) and an inner cellular perineurium (Wigglesworth, 1959b, 1960; Scharrer, 1939; Twarog and Roeder, 1956). The perineurium

appears to be less permeable than the neural lamella (Wigglesworth, 1959a, 1960). The only tissue in which ionic regulation has been studied in any detail so far is the nerve cord of the cockroach *Periplaneta americana*.

Following the injection of large amounts of KCl into the gut, Tobias (1948a) observed in *Periplaneta* a threefold increase in the concentration of haemolymph potassium, a 56% increase in the concentration of nerve-cord potassium, and a slight drop in nerve-cord water; there was

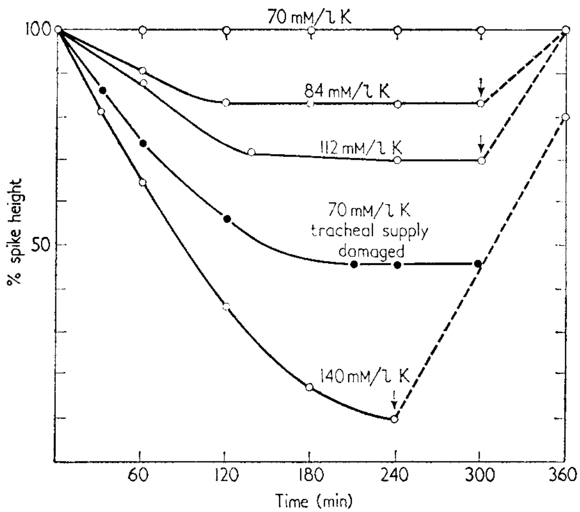

FIG. 16. The relationship between the spike height of a single motor axon of the crural nerve and the concentration of potassium in the bathing fluid. Recovery following upon bathing in 10 mM/l K (starting at the arrows) is indicated by the dotted lines. Typical results from five locusts. From Hoyle, 1953.

no significant change in muscle potassium nor in nerve-cord sodium, but a drop of 29% in the concentration of muscle sodium was observed. Hoyle (1953, 1954) studied the effect of an increased concentration of K^+ upon the functioning of nerve and muscle in *Locusta migratoria migratorioides*. The nerves were exposed *in situ* and irrigated with saline solutions approximating in composition to the haemolymph. The crural nerve was not readily depolarized by an increased external potassium concentration: it was unaffected by 70 mM/l potassium over a period of 6 h, but this concentration had a marked effect when the tracheal supply was interrupted (Fig. 16). When injected beneath the nerve sheath 50–70 mM/l K^+ produced depolarization within a few seconds. The

effect of high K^+ was also very rapid when applied to the partially desheathed nerve, and in these cases recovery upon irrigating with normal saline was very quick, in contrast to the situation in the normal nerve (Fig. 16). Muscle, which lacks a cellular sheath comparable to the perineurium, was found to behave towards high K^+ rather like desheathed nerve. Similar results (Table XX) were obtained from the connectives of the abdominal nerve cord of *Periplaneta americana*

TABLE XX

The effect of high concentrations of potassium upon the activity of the abdominal nerve cord of *Periplaneta americana* (from Twarog and Roeder, 1956)

Solution	Blocking time	Recovery time	Type of preparation
	A. NERVOUS SHEATH INTACT		
*180 mM/l K	12 min	10 min	Connective only
*180 mM/l K	15 min	10 min	Ganglion included
*180 mM/l K	18 min	20 min	Ganglion included
†140 mM/l K	30 min	25 min	Ganglion included
†140 mM/l K	22 min	33 min	Ganglion included
	B. RECORDING ELECTRODES ON DESHEATHED SEGMENT		
*180 mM/l K	10 sec	4 min	Ganglion included
†140 mM/l K	60 sec	2 min	Ganglion included
†140 mM/l K	90 sec	2 min	Ganglion included

* 180 mM/l KCl.
† Balanced saline solution of Hoyle (1953). Recovery was followed in K^+ free saline.

(Twarog and Roeder, 1956), though the nerves of this species are considerably more sensitive to high K^+ than those of *Locusta*. In the presence of the sheath the nerve cord can function for several hours in a sodium-deficient saline (Twarog and Roeder, 1956) and in isotonic glucose (Yamasaki and Narahashi, 1959a), and the effect of high concentrations of acetyl choline upon synaptic transmission in the last abdominal ganglion is reduced (Twarog and Roeder, 1956). These authors all interpreted their results in terms of the sheath acting as a diffusion barrier, and in addition Hoyle (1953) suggested that the sheath might do work to oppose the influx of potassium.

Wigglesworth (1959b, 1960) pointed out that nutritive and other substances must reach the nervous tissue by diffusion through the

sheath as the tissue is avascular, and Treherne (1960) found a rapid metabolism and exchange of sugars between the haemolymph and abdominal nerve cord, the influx of sugars into the nerve cord being equivalent to 1·1 mM glucose/l of nerve cord water/min. Subsequent studies (Treherne 1961a–d), conducted both *in vivo* and *in vitro*, showed that the exchange of sodium and potassium between the haemolymph and the nerve cord was rapid, the sodium half-exchange time ($T_{\frac{1}{2}}$) being about 5 min in isolated abdominal nerve cords, connectives and terminal abdominal ganglia. The exponential loss of radio-sodium from these structures is much reduced by dilute KCN, or 2,4 dinitrophenol, or by potassium-free saline, but is unaltered by sodium-free saline. On the basis of these results Treherne suggested that an active secretory mechanism which is associated with the uptake of potassium is responsible for the extrusion of sodium from the nervous tissue (cf. Harris, 1954; Hodgkin and Keynes, 1954), and that the exchanges of sodium are not attributable to exchange diffusion. Removal of about 50% of the sheath of terminal abdominal ganglia had no noticeable effect on the $T_{\frac{1}{2}}$ of sodium exchange, and the $T_{\frac{1}{2}}$ for these structures is similar to those obtained from isolated nerve cords and connectives, which, considering the differences in surface/volume ratio, suggests that extrusion of sodium across the membranes of the underlying cells may be the rate limiting process in the exchange. Further inquiry showed that the efflux of radio-sodium from isolated nerve cords could, over the first 100–200 sec, be resolved into a fast component, (complex initially, later exponential; $T_{\frac{1}{2}}$ 29 sec) and a slow exponential component equivalent to that measured in earlier experiments. Similar findings were made with terminal abdominal ganglia, and here partial desheathing had no effect upon the fast component. The initial complexity of the fast components is no doubt partly due to washing off surface radioactivity. A later investigation (Treherne, 1962) showed (by means of H^3OH) that 21·6% of the nerve cord water exchanged rapidly, and this was in good agreement with a measured inulin space of 18·2%. This fraction was identified as the extracellular water. Rapidly exchanging fractions of the total K, Ca and Cl in the nerve cord were found in addition to the rapidly exchanging fraction of Na. These fractions were considered to be located in the extracellular water (Treherne, 1961d, 1962), and their estimated concentrations in this water are shown in Table XXI. From the ratios given in Table XXI it appears that Na, K, Ca and Cl may be distributed between the haemolymph and extracellular water in accordance with a Donnan equilibrium arising from the presence in the extracellular water of an anion unable to diffuse

through the nerve sheath. When terminal abdominal ganglia were desheathed it was found that the extracellular water was roughly trebled, and that the concentrations of sodium and calcium in this water fell to the levels of the outside solution. Treherne therefore suggested that the increased potassium depolarization following upon desheathing (Hoyle, 1953; Twarog and Roeder, 1956) may result from the disturbance of the Donnan equilibrium; this entails amongst other things a

TABLE XXI

The distribution of Na, K, Ca and Cl between bathing solution, extracellular water, and cellular components, for nervous tissue of *Periplaneta americana* (from Treherne, 1962)

Ion	Concentration in outside solution mM/l	Concentration in extracellular water mM/l	Concentration in cellular components mM/l tissue water	Ionic ratios
Na	157	(255) 284	(101)	$\frac{\text{Na out}}{\text{Na extracellular}} = 0{\cdot}554$
K	12·3	17·1	approx. 200*	$\frac{\text{K out}}{\text{K extracellular}} = 0{\cdot}72$
Ca	4·5	17·6	at least 14·7	$\frac{(\text{Ca out})^{\frac{1}{2}}}{(\text{Ca extracellular})^{\frac{1}{2}}} = 0{\cdot}506$
Cl	184	107	—	$\frac{\text{Cl extracellular}}{\text{Cl out}} = 0{\cdot}58$

Figures in brackets are for terminal abdominal ganglia, the others are for whole abdominal nerve cords.
* Calculated using data for whole abdominal nerve cords (Treherne, 1961a).

fourfold drop in the calcium concentration of the extracellular water, and it has been shown by Stämpfli and Nishie (1956) that a reduction in calcium concentration enhances potassium depolarization in frog nerve. The presence of a Donnan equilibrium between haemolymph and extracellular water might account for the prolonged functioning of normal nerve in isotonic glucose found by Yamasaki and Narahashi (1959a), but it is difficult to see how a simple Donnan equilibrium could account for the prolonged functioning in sodium-deficient saline (sodium largely replaced by glucose) found by Twarog and Roeder (1956), as one would expect the sodium in the extracellular water to come into equilibrium with that in the bathing fluid. This expectation is supported

by Treherne's finding (Treherne, 1962) that when the sodium in the bathing solution is reduced from 157 to 12·3 mM/l (being replaced by corresponding amounts of potassium) the sodium in the rapidly exchanging fraction is reduced to about one-sixteenth of the normal value. Since the giant axons of *Periplaneta* seem to function after the manner of squid axons (Yamasaki and Narahashi, 1959a, b) a sodium concentration in the extracellular water higher than that in the nervous tissue must be maintained, and it seems possible that a sodium pump directed to this end might occur in the nervous sheath of those insects which have a low concentration of sodium in their haemolymph.

VII. The Overall Regulation of Salt and Water Balance in Insects

The effectiveness of the overall regulation of the ionic composition of the haemolymph in freshwater and sea-water insects during relatively large changes in the composition of the external medium is well established. It is probable that the regulatory ability of many terrestrial insects is also well developed, although systematic investigations on these animals—for example, by providing fasting animals with solutions of varying ionic composition to drink—are necessary in order to establish the effectiveness of the regulatory mechanisms.

The main sites for the exchange of water and salts in insects are now known and it is thus possible to set up equations for the maintenance of ion and water balance for an insect in any of the three major environments. Thus in a salt-water insect, which maintains its haemolymph hypo-osmotic to the external medium, for any of the haemolymph ions:

$$M^{g}_{in} + (M^{p}_{in} - M^{p}_{out}) = M^{u}_{out} \qquad (1)$$

where M^{g}_{in} is the net flux of the ion through the mid-gut wall, M^{p}_{in} is the passive influx through the integument, M^{p}_{out} is the passive efflux, and M^{u}_{out} is the efflux of the ion through the excretory system. For water:

$$V^{g}_{in} = (V^{p}_{out} - V^{p}_{in}) + V^{u}_{out} \qquad (2)$$

where V^{g}_{in}, V^{p}_{out}, V^{p}_{in} and V^{u}_{out} are the equivalent water fluxes.

From equations (1) and (2) it follows that for the maintenance of a constant concentration of the ion in the haemolymph:

$$\frac{M^{g}_{in} + (M^{p}_{in} - M^{p}_{out})}{V^{g}_{in} - (V^{p}_{out} - V^{p}_{in})} = \frac{M^{u}_{out}}{V^{u}_{out}} \qquad (3)$$

Equation (3) is also applicable to terrestrial insects (which are similar to the salt-water forms in their osmotic problems), except that the net passive flux of the ion through the integument, $(M^p_{in} - M^p_{out})$, is zero. Thus for both salt-water and terrestrial insects the concentration of the ion (M^u_{out}/V^u_{out}) in the rectal fluid must be at least equal to, and generally greater than, the concentration of the ion in the fluid absorbed from the mid-gut (M^g_{in}/V^g_{in}). It also follows that the regulation of the concentration of the ion in the haemolymph may depend on the regulation of both the water and the ion fluxes (i.e., the two cannot be considered independently). Since as far as is known the water and ion fluxes through the integument are determined solely by the environmental conditions (i.e., they are not themselves regulated—with the possible exception of the active uptake of water vapour in some terrestrial insects), regulation can only be effected by variations in the relative proportions of water and ions absorbed in the mid-gut or eliminated through the excretory system.

Unfortunately, the conditions governing the uptake of water and salts in the mid-gut are not known. The fact that salt-water insects can keep in water balance by drinking, and also that *Schistocerca*, at 70% R.H., can maintain its haemolymph volume if supplied with a hyperosmotic saline solution to drink (Phillips, 1961), suggest that water can be absorbed from hyperosmotic solutions, but the mechanism is unknown. Measurements of the osmotic pressure of the mid-gut fluid in *Aëdes detritus* larvae from sea water indicate that the absorption process may be complex. Ramsay (1950) found that the mid-gut fluid was only slightly more concentrated than the haemolymph and that the fluid from the mid-gut caeca, although more concentrated, was still well below that of the sea water. Since swallowed sea water presumably first enters the mid-gut, a process of osmotic equilibration must take place here. In *A. aegypti* larvae the mid-gut fluid passes forwards to the mid-gut caeca where absorption appears to take place (Wigglesworth, 1933b; Ramsay, 1950)—if the same events occur in *A. detritus* then the absorbed fluid must be less concentrated than that of the sea-water itself. However providing the mid-gut fluid is not regurgitated or passed through the hind-gut after osmotic equilibration, the overall process of absorption can still result in the uptake of a solution of the same concentration as the swallowed sea water. There is no evidence that the overall absorption of fluid in the insect mid-gut can concentrate fluid in the gut by the uptake of a less concentrated solution.

Further studies on the uptake of water and ions by the mid-gut are highly desirable. The technique devised by Treherne (1958, 1959) for

the study of the uptake of sugars and amino-acids in the locust gut should be readily adaptable for this purpose. A comparison of the osmotic pressure of the rectal fluid of salt-water insects with that of their external medium (Ramsay, 1950; Sutcliffe, 1960), and of the ionic composition of the rectal fluid of saline-fed *Schistocerca* with that of the saline solution (Phillips, 1961; Tables XVI and XVIII), suggests that the requirement that the concentration in the rectal fluid is not less than that of the fluid absorbed in the mid-gut is approximately met—on the assumption that this fluid has the same composition as that swallowed. It is not known whether the relative proportions of water and ions in the fluid absorbed in the mid-gut can be regulated in relation to the salt and water requirements of the insect. If this is not possible (i.e., if the fluid in the mid-gut is always absorbed as such), then the whole burden of regulation of the ionic composition of the haemolymph in salt-water and terrestrial insects must fall upon the excretory system—a system which is well adapted for this rôle.

Since the fluid eliminated by the excretory system consists of the Malpighian tubule secretion modified by the reabsorption of water and ions in the hind-gut, the concentration of an ion in the rectal fluid can be written:

$$\frac{M^u_{\text{out}}}{V^u_{\text{out}}} = \frac{M^s - M^r}{V^s - V^r} \tag{4}$$

where M^s and V^s are the respective rates of secretion of the ion and of water by the Malpighian tubules, and M^r and V^r are their respective rates of reabsorption after $M^u_{\text{out}}/V^u_{\text{out}}$ has reached its steady state value. Now it might have been supposed that, just as the secretion of water into the Malpighian tubule is closely linked with active potassium transport, water reabsorption is also linked with the reabsorption of potassium (and other ions). But in *Schistocerca* this is clearly not the case—independent active transport systems for water and ions are present in the rectum (Phillips, 1961). The independence of salt and water reabsorption gives much greater freedom to the regulatory mechanism, since the uptake of water by active ion transport (which may move water against an osmotic gradient) could not result in an increase of the osmotic pressure of the rectal contents. An independent water uptake mechanism must be present in all insects which produce a hyperosmotic rectal fluid by reabsorption from the primarily iso-osmotic Malpighian tubule fluid.

Equation (4) shows that by suitable adjustment of M^r and V^r—and regulation of both is known to occur in the rectum of *Schistocerca*

(Phillips, 1961)—the rectal fluid volume and concentration can be varied widely within the limits set by the rate of Malpighian tubule secretion. Adjustment of M^r and V^r might be achieved either by altering the active transport rate or by changing the passive permeability of the rectal wall. There is, however, a practical limitation to the concentration of any ion which can be attained in the rectum: the minimum concentration is determined by the gradient against which ion absorption can take place and, at the same time, the extent to which water uptake can be reduced; the maximum concentration is determined by the osmotic gradient against which water can be absorbed and the extent to which ion absorption can be reduced. Nevertheless the two concentrations may be widely separated—for example, in *Schistocerca* the chloride concentration can be varied between 5 and 569 mM/l and sodium between 1 and 405 mM/l (see Table XVIII); the salt-water insects also show a wide range in the osmotic pressure of the rectal fluid (Ramsay, 1950; Sutcliffe 1960)—so that the potentialities for regulation by the excretory system are very great.

The Malpighian tubules themselves probably play rather a minor rôle in regulation. In the isolated tubules of *Dixippus* the rate of secretion is little changed by variations in the ionic composition of the medium (except for potassium), and *in vivo* there is little difference between feeding and fasting animals (Ramsay, 1955a, b; 1956). In *Schistocerca* the rate of secretion is practically unchanged in water-fed and saline-fed animals (Phillips, 1961—Table XVIII). The only known case where the rate of tubular secretion varies greatly is in *Rhodnius*, where diuresis occurs for a few hours after a blood meal (Wigglesworth, 1931a; Ramsay, 1952; Maddrell, 1962)—but this may well be true of other blood-sucking insects.

The four variables, M^s, V^s, M^r, and V^r (in equation (4)), then, may all be involved to a greater or lesser extent in determining the composition of the fluid eliminated by the excretory system, but at present the internal factors which control these variables *in vivo* are not fully known. The osmotic pressure of the haemolymph is a factor of obvious importance, but the possibility that the haemolymph volume and the concentration of individual ions are also involved must not be overlooked. The demonstration by Maddrell (1962) of the presence of a hormone which determines the rate of Malpighian tubule secretion in *Rhodnius* suggests that other hormones which control rectal reabsorption may also be found.

In freshwater insects the situation is essentially analagous to that of salt-water and terrestrial forms, the main difference being that the net

passive fluxes for both water and ions ($V^p_{out} - V^p_{in}$; $M^p_{in} - M^p_{out}$; in equation (3)) are in the opposite direction. Since the animals have a tendency to lose salts and gain water, the excretory system is normally adjusted to produce a relatively large volume of rectal fluid containing very low concentrations of the haemolymph ions. Regulation by the excretory system in these forms is therefore restricted to preventing an increase in the haemolymph ion concentration and is limited by the fact that the rectal fluid osmotic pressure cannot exceed that of the haemolymph. Many fresh-water insects do not rely on the uptake of ions in the mid-gut to balance the total loss but possess active transport systems capable of extracting ions from the external medium. In the larvae of *Aëdes aegypti* there is good evidence of the physiological control of the sodium uptake mechanism (Stobbart, 1960; Fig. 8) although, again, the internal factors involved in the control are not fully understood.

The evidence, then, favours the view that many aquatic insects and some terrestrial ones are able to regulate the composition of their haemolymph rather closely, and thus, like many other animals, have achieved a high degree of independence from their external environment. On the other hand, in some terrestrial insects regulation may not be so effective; and as an example the relatively ineffective regulation of haemolymph sodium in *Dixippus* may be quoted (Ramsay, 1955a). Sluggish regulation may be expected in any terrestrial insect where the regulatory potentialities of the excretory system are limited by lack of water or by the attainment of a relatively low osmotic pressure in the rectal fluid. Nevertheless these insects may be more tolerant of changes in the concentration of ions in the haemolymph. Thus in *Dixippus* the injection of enough NaCl to raise the haemolymph sodium to 4–5 times the normal value appears to be without effect on the animals (Ramsay, 1955a) and, similarly, the locust is able to tolerate considerable changes in the haemolymph potassium concentration (Hoyle, 1954). Although there is no direct correlation between haemolymph composition and diet, as was originally supposed (Boné, 1944), there is no doubt that in many insect Orders the haemolymph ionic ratios bear a closer resemblance to the dietary ratios than to the ratio of ions in the haemolymph of the more primitive forms (Tables I, X and XI). Adaptation to a highly abnormal haemolymph composition may be the result of (*a*) the tolerance of the cells to a high degree of variability in the ionic make-up of the haemolymph, or their specific adaption to a particular composition, or (*b*) the establishment of local tissue environments. Evidence for the first possibility comes from the fact that the isolated tubules of *Dixippus* continue to function in a medium of grossly abnormal ionic

composition providing some haemolymph is present (Ramsay, 1955b, 1956). Also, in attempts to culture insect cells in isolation, success appears to be more dependent on the presence of unknown haemolymph factors than on the careful control of the ionic composition of the medium (see Day and Grace, 1959; Grace, 1962). Similarly, the isolated heart of the pupae of the cynthia moth, *Samia walkeri*, continues to beat for 24 h after immersion in a saline solution with a Na/K ratio of 1 : 13·8—but the sodium content can be increased so that the ratio is 34 : 1 without adverse effects (Barsa, 1955). There is some evidence that the striated muscle fibres of *Dixippus* are adapted to function in a haemolymph of abnormal composition and that neuro-muscular transmission is effected in a different manner from that in many other muscle cells (Wood, 1957). In vertebrate muscle fibres the presence of sodium ions in the blood is necessary for the complete development of the end-plate potential and for the production of the active membrane response (Hodgkin, 1951; Fatt and Katz, 1952)—and the same situation appears to exist in the leg muscles of *Locusta migratoria* and *Periplaneta americana* (Wood, 1961). However, in *Dixippus* the absence of sodium, although decreasing the resting and action potentials slightly, does not prevent the development of an action potential. It is probable that here sodium ions are not the specific carriers of the action current. The action potential declines when the magnesium concentration is reduced below 50 mM/l and it is possible that these ions contribute significantly to the action current (Wood, 1957).

Conclusive evidence of the importance of local tissue environments is not yet forthcoming. Interest centres around the nervous system (Hoyle, 1953) since it has yet to be established that propagated action potentials can be produced in insect nerve axons in a medium with a Na/K ratio below unity. In *Periplaneta* it appears that the nerve sheath does enclose a medium surrounding the nerve cells of a somewhat different composition from that of the haemolymph, although it is probably maintained passively (Treherne, 1962). It is not impossible that in insects with a low Na/K ratio in the haemolymph this situation is exploited by the incorporation of active transport mechanisms in the nerve sheath which can maintain a suitable ionic environment for the nerve cells.

Acknowledgements

We are indebted to Dr. J. E. Phillips for permission to quote from his thesis and to reproduce three figures from it, and to Drs. B. W. Staddon and D. W. Sutcliffe for permission to quote unpublished observations.

REFERENCES

Andersen, B. and Ussing, H. H. (1960). *In* "Comparative Biochemistry", Vol. II (Florkin, M. and Mason, H. S., eds). Academic Press, London. Active transport.

Barsa, M. C. (1955). *J. gen. Physiol.* **38**, 79–92. The behaviour of isolated hearts of the grasshopper *Chortophaga viridifasciata* and the moth *Samia walkeri* in solutions with different concentrations of sodium potassium calcium and magnesium.

Beadle, L. C. (1939). *J. exp. Biol.* **16**, 346–62. Regulation of the haemolymph in the saline water mosquito larva *Aëdes detritus* Edw.

Beadle, L. C. (1943). *Biol. Rev.* **18**, 172–83. Osmotic regulation and the fauna of inland waters.

Beadle, L. C. and Shaw, J. (1950). *J. exp. Biol.* **27**, 96–109. The retention of salt and the regulation of the non-protein nitrogen fraction in the blood of the aquatic larva (*Sialis lutaria*).

Beament, J. W. L. (1954). *Symp. Soc. exp. Biol.* **8**, 94–117. Water transport in insects.

Beament, J. W. L. (1958). *J. exp. Biol.* **35**, 494–519. The effect of temperature on the waterproofing mechanism of an insect.

Beament, J. W. L. (1959). *J. exp. Biol.* **36**, 391–422. The waterproofing mechanism of arthropods. I. The effect of temperature on cuticle permeability in terrestrial insects and ticks.

Beament, J. W. L. (1961a). *Biol. Rev.* **36**, 281–320. The water relations of insect cuticle.

Beament, J. W. L. (1961b). *J. exp. Biol.* **38**, 277–90. The waterproofing mechanism of arthropods. II. The permeability of the cuticle of some aquatic insects.

Boné, G. J. (1944). *Ann. Soc. zool. Belg.* **75**, 123–32. Le rapport sodium/potassium dans le liquide coelomique des insects. 1. Ses relations avec le régime alimentaire.

Boné, G. J. (1947). *Nature, Lond.* **160**, 679. Regulation of the sodium/potassium ratio in insects.

Boné, G. and Koch, H. J. (1942). *Ann. Soc. zool. Belg.* **73**, 73–87. Le Rôle des Tubes de Malpighi et du Rectum dans la Régulation Ionique chez les Insectes.

Browning, T. O. (1954). *J. exp. Biol.* **31**, 331–40. Water balance in the tick *Ornithodorus moubata* Murray, with particular reference to the influence of carbon dioxide on the uptake and loss of water.

Bryan, G. W. (1960). *J. exp. Biol.* **37**, 100–12. Sodium regulation in the crayfish *Astacus fluviatilis*. II. Experiments with sodium depleted animals.

Buck, J. B. (1953). *In* "Insect Physiology" (Roeder, K. D., ed.). John Wiley & Sons, New York. Physical properties and chemical composition of insect blood.

Buxton, P. A. (1930). *Proc. roy. Soc.* B **106**, 560–77. Evaporation from the mealworm and atmospheric humidity.

Clark, E. W. (1958). *Ann. ent. Soc. Amer.* **51**, 142–54. A review of literature on calcium and magnesium in insects.

Clark, E. W. and Craig, R. (1953). *Physiol. Zoöl.* **26**, 101–7. The calcium and magnesium content in the haemolymph of certain insects.

Claus, A. (1937). *Zool Jb.* **58**, 365–432. Vergleichend-physiologische Untersuchungen zur Oekologie der Wasserwanzen, mit besonderer Berücksichtigung der Brackwasserwanze *Sigara lugubris* (Fieb).

Croghan, P. C. (1958a). *J. exp. Biol.* **35,** 219–33. The osmotic and ionic regulation of *Artemia salina* (L).
Croghan, P. C. (1958b). *J. exp. Biol.* **35,** 425–36. Ionic fluxes in *Artemia salina* (L).
Croghan, P. C. and Lockwood, A. P. M. (1960). *J. exp. Biol.* **37,** 339–43. The composition of the haemolymph of the larva of *Drosophila melanogaster.*
Day, M. E. and Grace, T. D. C. (1959). *Annu. Rev. Ent.* **4,** 17–38. Culture of insect tissues.
Diamond, J. (1962). *J. Physiol.* **161,** 503–27. The mechanism of water transport by the gall bladder.
Duchâteau, G., Florkin, M. and Leclercq, J. (1953). *Arch. int. Physiol.* **61,** 518–49. Concentrations des bases fixes et types de compositions de la base totale de l'hémolymphe des Insectes.
Durbin, R. P. (1960). *J. gen. Physiol.* **44,** 315–26. Osmotic flow of water across permeable cellulose membranes.
Edney, E. B. (1947). *Bull. ent. Res.* **38,** 263–80. Laboratory studies on the bionomics of the rat fleas *Xenopsylla brasiliensis* Baker and *X. cheopis* Roths. II. Water relations during the coccoon period.
Edney, E. B. (1957). "The water relations of terrestrial arthropods". Cambridge Univ. Press, London.
Fatt, P. and Katz, B. (1952). *J. Physiol.* **118,** 73–87. The effect of sodium ions on neuromuscular transmission.
Gloor, H. and Chen, P. S. (1950). *Rev. suisse Zool.* **57,** 570. Über ein Analorgan bei *Drosophila*-Larven.
Govaerts, J. and Leclercq, J. (1946). *Nature, Lond.* **157,** 483. Water exchange between insects and air moisture.
Grace, T. D. C. (1962). *Nature, Lond.* **195,** 788–89. Establishment of four strains of cells from insect tissues grown *in vitro.*
Harris, E. J. (1954). *Symp. Soc. exp. Biol.* **8,** 228–41. Linkage of sodium and potassium active transport in human erythrocytes.
Hassett, C. C. and Jenkins, D. W. (1951). *Physiol. Zoöl.* **24,** 257–66. The uptake and effect of radiophosphorus in mosquitoes.
Hodgkin, A. L. (1951). *Biol. Rev.* **26,** 339–409. The ionic basis of electrical activity in nerve and muscle.
Hodgkin, A. L. and Keynes, R. D. (1954). *Symp. Soc. exp. Biol.* **8,** 423–37. Movement of cations during recovery in nerve.
Holdgate, M. W. (1956). *J. exp. Biol.* **33,** 107–18. Transpiration through the cuticles of some aquatic insects.
Hoyle, G. (1953). *J. exp. Biol.* **30,** 121–35. Potassium ions and insect nerve muscle.
Hoyle, G. (1954). *J. exp. Biol.* **31,** 260–70. Changes in the blood potassium concentration of the African migratory locust (*Locusta migratoria migratorioides* R. and F.) during food deprivation, and the effect of neuromuscular activity.
Jørgensen, C. B., Levi, H. and Zerahn, K. (1954). *Acta. physiol. scand.* **30,** 178–90. On active uptake of sodium and chloride in anurans.
Koch, H. J. (1938). *J. exp. Biol.* **15,** 152–60. The absorption of chloride ions by the anal papillae of diptera larvae.
Koch, H. J. (1954). *Arch. int. Physiol.* **62,** 136. L'intervention de cholinesterases dans l'absorption et le transport actif de matières minérales par les branchies du crabe *Eriocheir sinensis* (M. Edw.).

Koefoed Johnsen, V. and Ussing, H. H. (1953). *Acta physiol. scand.* **28,** 60–76. The contribution of diffusion and flow to the passage of D_2O through living membranes.

Krogh, A. (1939). "Osmotic regulation in aquatic animals". Cambridge Univ. Press, London.

Leclercq, J. (1946). *Bull. Ann. Soc. ent. Belg.* **82,** 71–5. Des insectes qui boivent de l'eau.

Lees, A. D. (1946). *Parasitology* **37,** 1–20. The water balance in *Ixodes ricinus* L. and certain other species of ticks.

Levenbook, L. (1950). *Biochem. J.* **47,** 336–46. The composition of horse bot fly (*Gastrophilus intestinalis*) larva blood.

Ludwig, D. (1937). *Physiol. Zoöl.* **10,** 342–51. The effect of different relative humidities on respiratory metabolism and survival of the grasshopper *Chortophaga viridifasciata* de Geer.

Ludwig, D. (1951). *Physiol. Zoöl.* **24,** 329–34. Composition of the blood of the Japanese beetle (*Popillia japonica* Newman).

Maddrell, S. H. P. (1962). *Nature, Lond.* **194,** 605–6. A diuretic hormone in *Rhodnius prolixus* Stål.

Mellanby, K. (1932). *Proc. roy. Soc.* B. **111,** 376–90. The effect of atmospheric humidity on the metabolism of the fasting mealworm (*Tenebrio molitor* L., Coleoptera).

Mellanby, K. (1934). *Proc. roy. Soc.* B. **116,** 139–49. The site of water loss from insects.

Mellanby, K. (1939). *Biol. Rev.* **14,** 243–60. The functions of insect blood.

Meschia, G. and Setnikar, I. (1959). *J. gen. Physiol.* **42,** 429–44. Experimental study of osmosis through a collodion membrane.

Mitchell, P. (1954). *Symp. Soc. exp. Biol.* **8,** 254–61. Transport of phosphate through an osmotic barrier.

Nemenz, H. (1960a). *J. Insect. Physiol.* **4,** 38–44. On the osmotic regulation of the larvae of *Ephydra cinerea.*

Nemenz, H. (1960b). *S.B. öst. Akad. Wiss.* **169,** 17–41. Experimente zur Ionenregulation der Larve von *Ephydra cinerea* Jones (Dipt.).

Neumann, D. (1961). *Biol. Zbl.* **80,** 693–715. Osmotische Resistenz und Osmoregulation aquatischer Chironomidenlarven.

Patton, R. L. and Craig, R. (1939). *J. exp. Zool.* **81,** 437–57. The rates of excretion of certain substances by the larvae of the mealworm, *Tenebrio molitor* L.

Phillips, J. E. (1961). "Rectal absorption of water and salts in the locust and blowfly". Ph.D. Thesis, University of Cambridge.

Quintart, A. (1961). *Ann. Soc. zool. Belg.* **91,** 117–28. L'organe anal de la larve de *Drosophila melanogaster* (Meig.).

Ramsay, J. A. (1935). *J. exp. Biol.* **12,** 373–83. The evaporation of water from the cockroach.

Ramsay, J. A. (1950). *J. exp. Biol.* **27,** 145–57. Osmotic regulation in mosquito larvae.

Ramsay, J. A. (1951). *J. exp. Biol.* **28,** 62–73. Osmotic regulation in mosquito larvae: the rôle of the Malpighian tubules.

Ramsay, J. A. (1952). *J. exp. Biol.* **29,** 110–26. The excretion of sodium and potassium by the Malpighian tubules of *Rhodnius.*

Ramsay, J. A. (1953a). *J. exp. Biol.* **30,** 79–89. Exchanges of sodium and potassium in mosquito larvae.

Ramsay, J. A. (1953b). *J. exp. Biol.* **30,** 358–69. Active transport of potassium by the Malpighian tubules of insects.

Ramsay, J. A. (1954). *J. exp. Biol.* **31,** 104–13. Active transport of water by the Malpighian tubules of the stick insect *Dixippus morosus* (Orthoptera, Phasmidae).

Ramsay, J. A. (1955a). *J. exp. Biol.* **32,** 183–99. The excretory system of the stick insect *Dixippus morosus* (Orthoptera, Phasmidae).

Ramsay, J. A. (1955b). *J. exp. Biol.* **32,** 200–16. The excretion of sodium, potassium and water by the Malpighian tubules of the stick insect *Dixippus morosus* (Orthoptera, Phasmidae).

Ramsay, J. A. (1956). *J. exp. Biol.* **33,** 697–709. Excretion by the Malpighian tubules of the stick insect *Dixippus morosus* (Orthoptera, Phasmidae): calcium, magnesium, chloride, phosphate and hydrogen ions.

Ramsay, J. A. (1958). *J. exp. Biol.* **35,** 871–91. Excretion by the Malpighian tubles of the stick insect *Dixippus morosus* (Orthoptera, Phasmidae): amino acids, sugars and urea.

Richards, A. G. (1951). "The Integument of Arthropods". University of Minnesota Press, Minneapolis.

Richards, A. G. (1958). *Ergebn. Biol.* **20,** 1–26. The cuticle of arthropods.

Roeder, K. D. (Ed.) (1953). "Insect Physiology". John Wiley & Sons, New York.

Rosenberg, T. (1954). *Symp. Soc. exp. Biol.* **8,** 27–41. The Concept and Definition of Active Transport.

Rouschal, W. (1940). *Z. wiss Zool.* A **153,** 196–218. Osmotische Werte wirbellose landtiere und ihre ökologische Bedeutung.

Schaller, F. (1949). *Z. vergl. Physiol.* **31,** 684–95. Osmoregulation und Wasserhaushalt der Larve von *Corethra plumicornis* mit besonderer Berücksichtigung der Vorgänge am Darmkanal.

Scharrer, B. C. J. (1939). *J. comp. Neurol.* **70,** 77–88. The differentiation between neuroglia and connective tissue sheath in the cockroach (*Periplaneta americana*).

Schoffeniels, E. (1950). *Arch. int. Physiol.* **58,** 1–4. La régulation de la pression osmotique et de la chlorémie chez les larves d'Odonates.

Schoffeniels, E. (1960). *Arch. int. Physiol.* **68,** 507–8. Rôle des acides aminés dans la régulation de la pression osmotique du milieu intérieur des Insectes aquatiques.

Shaw, J. (1955a). *J. exp. Biol.* **32,** 330–52. The permeability and structure of the cuticle of the aquatic larva of *Sialis lutaria.*

Shaw, J. (1955b). *J. exp. Biol.* **32,** 353–82. Ionic regulation and water balance in the aquatic larva of *Sialis lutaria.*

Shaw, J. (1959a). *J. exp. Biol.* **36,** 126–44. The absorption of sodium ions by the crayfish *Astacus pallipes* Lereboullet. I. The effect of the internal and external sodium concentrations.

Shaw, J. (1959b). *J. exp. Biol.* **36,** 157–76. Salt and water balance in the East African fresh-water crab *Potomon niloticus* (M. Edw.).

Shaw, J. (1960). *J. exp. Biol.* **37,** 557–72. The absorption of chloride ions by the crayfish *Astacus pallipes* Lereboullet.

Shaw, J. (1961). *J. exp. Biol.* **38,** 153–62. Sodium balance in *Eriocheir sinensis* (M. Edw.). The adaptation of the crustacea to fresh water.

Smith, H. W. (1956). "Principles of Renal Physiology". Oxford Univ. Press, New York.

Staddon, B. W. (1955). *J. exp. Biol.* **32,** 84–94. The excretion and storage of ammonia by the aquatic larva of *Sialis lutaria* (Neuroptera).

Staddon, B. W. (1962). Unpublished observations.

Stämpfli, R. and Nishie, K. (1956). *Helv. physiol. acta.* **14,** 93–104. Effects of calcium-free solutions on membrane potential of myelinated nerve fibres of the Brazilian frog *Leptodactylus ocellatus.*

Starling, E. H. (1956). "Principles of Human Physiology" (Lovatt Evans 12th Edn., 1956). J. A. Churchill, London.

Stobbart, R. H. (1959). *J. exp. Biol.* **36,** 641–53. Studies on the exchange and regulation of sodium in the larva of *Aëdes aegypti* (L). I. The steady-state exchange.

Stobbart, R.H. (1960). *J. exp. Biol.* **37,** 594–608. Studies on the exchange and regulation of sodium in the larva of *Aëdes aegypti* (L). II. The net transport and the fluxes associated with it.

Stobbart, R. H. (1962). Unpublished observations.

Sutcliffe, D. W. (1959). "Studies on the biology of some salt-marsh insects". Ph.D. Thesis, King's College University of Durham, England.

Sutcliffe, D. W. (1960). *Nature, Lond.* **187,** 331–2. Osmotic Regulation in the Larvae of some Euryhaline Diptera.

Sutcliffe, D. W. (1961a). *J. exp. Biol.* **38,** 501–19. Studies on salt and water balance in caddis larvae (Trichoptera). I. Osmotic and ionic regulation of body fluids in *Limnephilus affinis* Curtis.

Sutcliffe, D. W. (1961b). *J. exp. Biol.* **38,** 521–30. Studies on salt and water balance in caddis larvae (Trichoptera). II. Osmotic and ionic regulation of body fluids in *Limnephilus stigma* (Curtis) and *Anabolia nervosa* (Leach).

Sutcliffe, D. W. (1962a). *J. exp. Biol.* **39,** 141–60. Studies on salt and water balance in caddis larvae (Trichoptera). III. Drinking and excretion.

Sutcliffe, D. W. (1962b). *J. exp. Biol.* **39,** 325–43. The composition of haemolymph in aquatic insects.

Sutcliffe, D. W. (1962c). Unpublished observations.

Sutcliffe, D. W. (1963). The chemical composition of haemolymph in insects and some other arthropods, in relation to their phylogeny. (In preparation).

Tobias, J. M. (1948a). *J. cell. comp. Physiol.* **31,** 125–42. Potassium sodium and water interchange in irritable tissues and haemolymph of an omnivorous insect *Periplaneta americana.*

Tobias, J. M. (1948b). *J. cell. comp. Physiol.* **31,** 143–8. The high potassium and low sodium in the body fluid of a phytophagous insect, the silkworm *Bombyx mori,* and the change before pupation.

Treherne, J. E. (1954a). *Trans. R. ent. Soc. Lond.* **105,** 117–30. Osmotic regulation in the larvae of *Helodes* (Coleoptera Helodidae).

Treherne, J. E. (1954b). *J. exp. Biol.* **31,** 386–401. The exchange of labelled sodium in the larva of *Aëdes aegypti* (L).

Treherne, J. E. (1958). *J. exp. Biol.* **35,** 297–306. The absorption of glucose from the alimentary canal of the locust *Schistocerca gregaria* (Forsk).

Treherne, J. E. (1959). *J. exp. Biol.* **36,** 533–45. Amino acid absorption in the locust (*Schistocerca gregaria* Forsk).

Treherne, J. E. (1960). *J. exp. Biol.* **37,** 513–33. The nutrition of the central nervous system in the cockroach *Periplaneta americana* L. The exchange and metabolism of sugars.

Treherne, J. E. (1961a). *J. exp. Biol.* **38,** 315–22. Sodium and potassium fluxes in the abdominal nerve cord of the cockroach *Periplaneta americana* L.

Treherne, J. E. (1961b). *J. exp. Biol.* **38,** 629–36. The movement of sodium ions in the isolated abdominal nerve cord of the cockroach *Periplaneta americana* L.

Treherne, J. E. (1961c). *J. exp. Biol.* **38,** 729–36. The efflux of sodium ions from the last abdominal ganglion of the cockroach *Periplaneta americana* L.

Treherne, J. E. (1961d). *J. exp. Biol.* **38,** 737–46. The kinetics of sodium transfer in the central nervous system of the cockroach *Periplaneta americana* L.

Treherne, J. E. (1962). *J. exp. Biol.* **39,** 193–218. The distribution and exchange of some ions and molecules in the central nervous system of *Periplaneta americana* L.

Twarog, B. M. and Roeder, K. D. (1956). *Biol. Bull.* **111,** 278–86. Properties of the connective tissue sheath of the cockroach abdominal nerve cord.

Ussing, H. H. (1947). *Nature, Lond.* **160,** 262–3. Interpretation of the exchange of radiosodium in isolated muscle.

Ussing, H. H. (1948). *Cold. Spr. Harb. Symp. quant. Biol.* **13,** 193–200. The use of tracers in the study of active ion transport across animal membranes.

Ussing, H. H. (1949). *Acta. physiol. scand.* **19,** 43–56. The distinction by means of tracers between active transport and diffusion. The transfer of iodide across the isolated frog skin.

Ussing, H. H. (1952). *Advanc. Enzymol.* **13,** 21–65. Some aspects of the application of tracers in permeability studies.

Waddington, C. H. (1959). *Nature, Lond.* **183,** 1654. Canalisation of development and genetic assimilation of acquired characters.

Wigglesworth, V. B. (1931a). *J. exp. Biol.* **8,** 411–27. The physiology of excretion in a blood-sucking insect, *Rhodnius prolixus* (Hemiptera Reduviidae). I. Composition of the urine.

Wigglesworth, V. B. (1931b). *J. exp. Biol.* **8,** 428–42. The physiology of excretion in a blood-sucking insect, *Rhodnius prolixus* (Hemiptera Reduviidae). II. Anatomy and histology of the excretory system.

Wigglesworth, V. B. (1931c). *J. exp. Biol.* **8,** 443–51. The physiology of excretion in a bloodsucking insect, *Rhodnius prolixus* (Hemiptera Reduviidae). III. The mechanism of uric acid excretion.

Wigglesworth, V. B. (1932). *Quart. J. micr. Sci.* **75,** 131–50. On the function of the so-called "rectal glands" of insects.

Wigglesworth, V. B. (1933a). *J. exp. Biol.* **10,** 1–15. The effect of salts on the anal gills of the mosquito larva.

Wigglesworth, V. B. (1933b). *J. exp. Biol.* **10,** 16–26. The function of the anal gills of the mosquito larva.

Wigglesworth, V. B. (1933c). *J. exp. Biol.* **10,** 27–37. The adaptation of mosquito larvae to salt water.

Wigglesworth, V. B. (1935). *Proc. roy. Soc.* B **118,** 394–419. The regulation of respiration in the flea *Xenopsylla cheopis* Roths.

Wigglesworth, V. B. (1938). *J. exp. Biol.* **15,** 235–47. The regulation of osmotic pressure and chloride concentration in the haemolymph of mosquito larvae.

Wigglesworth, V. B. (1945). *J. exp. Biol.* **21,** 91–114. Transpiration through the cuticle of insects.

Wigglesworth, V. B. (1953). "The Principles of Insect Physiology", 5th edn. Methuen, London.

Wigglesworth, V. B. (1956). *Quart. J. micr. Sci.* **97**, 89–98. The haemocytes and connective tissue formation in an insect *Rhodnius prolixus* (Hemiptera).

Wigglesworth, V. B. (1957). *Annu. Rev. Ent.* **2**, 37–54. The physiology of insect cuticle.

Wigglesworth, V. B. (1959a). *Quart. J. micr. Sci.* **100**, 285–98. The histology of the nervous system of an insect *Rhodnius prolixus* (Hemiptera). I. The peripheral nervous system.

Wigglesworth, V. B. (1959b). *Quart. J. micr. Sci.* **100**, 299–313. The histology of the nervous system of an insect *Rhodnius prolixus* (Hemiptera). II. The central ganglia.

Wigglesworth, V. B. (1960). *J. exp. Biol.* **37**, 500–12. The nutrition of the central nervous system in the cockroach *Periplaneta americana* L. The rôle of the perineurium and glial cells in the mobilisation of reserves.

Wigglesworth, V. B. and Gillett, J. D. (1936). *Proc. R. ent. Soc. Lond.* (A) 11, 104–7. The loss of water during ecdysis in *Rhodnius prolixus* Stål (Hemiptera).

Wigglesworth, V. B. and Salpeter, M. M. (1962). *J. Insect Physiol.* **8**, 299–307. Histology of the Malpighian tubules in *Rhodnius prolixus* Stål (Hemiptera).

Wood, D. M. (1957). *J. Physiol.* **138**, 119–39. The effect of ions upon neuromuscular transmission in a herbivorous insect.

Wood, D. M. (1961). *Comp. Biochem. Physiol.* **4**, 42–6. The effect of sodium ions on the resting and action potentials of locust and cockroach muscle fibres.

Wyatt, G. R. (1961). *Annu. Rev. Ent.* **6**, 75–102. The biochemistry of insect haemolymph.

Yamasaki, T. and Narahashi, T. (1959a). *J. Insect Physiol.* **3**, 146–58. The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon.

Yamasaki, T. and Narahashi, T. (1959b). *J. Insect Physiol.* **3**, 230–42. Electrical properties of the cockroach giant axon.

Zwicky, K. (1954). *Z. vergl. Physiol.* **36**, 367–90. Osmoregulatorische reaktionen der larve von *Drosophila melanogaster*.

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Functional Aspects of the Organization of the Insect Nervous System

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I. Introduction

An understanding of the physiology of the insect nervous system requires more than a knowledge of the various electrical and chemical events occurring there. The interpretation of the spatial relations of these processes and their integration within an extremely complex system depends upon an adequate knowledge of the structural organization of the system. During recent years studies have been made of the organization and fine structure of some insect nervous systems, together with some investigations on their physiology. This review is an attempt to correlate these two aspects of research in an endeavour to present a picture of the functional organization of the insect nervous system.

The nervous systems of insects are characterized by certain features which differ from those of the vertebrate animals which have been the principle objects of study in the past. There is, for example, no circulation of haemolymph in the insect nervous system. The ganglia of insects may be more than a millimetre in width and it might be expected, as Wigglesworth has suggested, that there would be some special provision for the transport of nutrient substances through these masses of tissue. Again, the haemolymph of certain insect species is known to have a

composition which differs from the blood plasma of vertebrate animals, in exhibiting relatively high concentrations of potassium and magnesium ions, and it has been concluded that some specialized mechanisms have been evolved to enable the axons to function in these peculiar chemical environments. It is clearly of importance, in consideration of any specialized physiological properties of the insect central nervous system, that an adequate structural background should be available, concerning the relationship between the components of the system, and between these and the extraganglionic medium.

A full analysis of the integrative functions of the central nervous system and of peripheral nerve conduction must ultimately involve a synthesis of information on the structural, electrical and chemical properties of the system. Up to the present time, knowledge of the first of these aspects has not kept pace with physiological advances: in particular, information on the precise relationship between nervous components and elements of the central and peripheral nerve sheath and neuroglial system has been scarce. In addition, the organization of the synaptic region of the insect central nervous system, the neuropile, has been little understood. The electron microscope has made possible the detailed structural characterization of these elements of the insect nervous system, and has afforded a basis for comparison and contrast with corresponding components of the nervous system of vertebrates and other animals. Certain functional aspects of recent structural findings are considered in the present account, since the value of any cytological study lies in defining more clearly the system with which the physiologist and biochemist is dealing, and as a corollary of this, in providing information on the functional significance of the details of organization of the cell types constituting the system.

II. The Nerve Sheath

Although it has been generally recognized that the neural and glial elements of the insect ganglion are contained within an envelope or sheath consisting of both a cellular and non-cellular layer, some confusion has arisen as a result of the variety of terms that have been used to denote these two components. Ashhurst (1959) describes the synonomy that has been used. Following the most common usage, the outer, non-cellular portion of the sheath will be referred to here as the "neural lamella". The underlying layer of cells has been termed the "perilemma" (Hoyle, 1952; Hess, 1958a) and the perineurium (Scharrer, 1939; Baccetti, 1955; Wigglesworth, 1956, 1959b, 1960a; Treherne,

1961a). The former term has, however, also been applied to the sheath as a whole (Scharrer, 1939; Johansson, 1957; Richards and Schneider, 1958; Pipa and Cook, 1958; Pipa *et al.*, 1959; etc.), while as Ashhurst (1959) and Hess (1958a) point out, the perineurium denotes a connective tissue layer in the vertebrate system. Ashhurst's alternative and purely descriptive term "sheath cell layer" has the advantage of being free from any implications concerning the homologies or function of this portion of the insect nervous system. However, the cellular layer in the ganglion differs cytologically from that ensheathing the connectives and peripheral nerves. In view of this confusion the term "perineurium" is retained, pending further clarification of the relationships between the various types of sheath cells throughout the nervous system.

The sheath surrounding the peripheral nerves and ganglia of insects has been pictured as acting as a selectively permeable barrier, separating the molecules and ions of the haemolymph from those of the nervous system and thus maintaining an independent ionic environment intimately surrounding the axons (Hoyle, 1952, 1953; Twarog and Roeder, 1956). However, the sheath surrounding the cockroach nervous system has recently been shown to be relatively permeable to ions and to admit molecules as large as inulin (Treherne, 1961e). In view of these two conflicting views, the structural characterization of the components of the sheath may be of importance in throwing light on its observed permeability properties.

A. THE NEURAL LAMELLA

A composite picture of the nature of the insect neural lamella has been built up through investigations on the histochemical, biochemical, X-ray diffraction and birefringence properties of this region, and also through structural studies employing electron microscopic techniques.

In the light microscope, the neural lamella has been described as being either homogeneous, as in *Blatella germanica*, *Galleria mellonella* (Pipa and Cook, 1958), *Locusta migratoria* (Ashhurst and Chapman, 1961) and *Periplaneta americana* (Scharrer, 1939; Ashhurst 1961b, c) or as exhibiting some fibrillar organization as in *Oncopeltus fasciatus* (Johansson, 1957; Pipa and Cook, 1958) and *Melophagus ovinus* (Pipa and Cook, 1958). Rudall (1955), in a survey of the occurrence of collagen in invertebrates, found "indications of the presence of collagen in the ventral nerve cord" of mantids in X-ray diffraction studies, while Baccetti (1956, 1957) obtained birefringence data suggesting the presence of a collagen-like material in the neural lamella of *Anacridium aegyptium*.

Richards and Schneider (1958) suggested that the low-intensity X-ray diffraction pattern afforded by the neural lamella material of *Periplaneta* likewise indicated the presence of a collagen but only to the extent of 10–20% of the total volume. This material was believed to constitute a longitudinally and circumferentially oriented system of small fibres, embedded in a non-fibrous matrix.

Histochemical investigation of the neural lamella of *Locusta* and *Periplaneta* was carried out by Ashhurst (1959, 1961b) who found that in each instance a collagen-like protein was present in association with a neutral mucopolysaccharide, and the resemblance of the former to the other vertebrate and invertebrate collagens was corroborated by the identification of substantial amounts of hydroxyproline in hydrolysates of isolated neural lamella material of *Locusta*.

Hess (1958a) showed that in electron micrographs of thin sections of osmium-fixed, phosphotungstic acid "stained" ganglia of *Periplaneta*, the neural lamella is revealed as a complex structure comprising a narrow outer region, homogeneous or finely granular in appearance, surrounding the main bulk of the sheath, which is largely filled with fibrils resembling collagen in being periodically banded. Hess noted a dense line at the base of the fibrous sheath, and he believed this to be the basement membrane of the underlying cell layer. The neural lamella of *Locusta* was found by Ashhurst and Chapman (1961) to resemble that of *Periplaneta* in its general organization; the banded fibrils appear to be arranged in layers parallel with the lamella surface and the preferred direction in which the fibrils lie within each layer varies throughout the structure. It would thus appear that the collagenous material is deployed in a multidirectional meshwork, with the long axes of the fibrils generally tangential with respect to the ganglion as a whole.

Certain features of the structure of the neural lamella surrounding the last abdominal ganglion of *Periplaneta* have been described in more detail (Treherne and Smith, in preparation) and the general conclusions of Hess, Ashhurst and Chapman have been substantiated. Figure 1 represents a peripheral area of the lamella in a transversely sectioned ganglion. The outer zone, here *c.* 0·3 μ in depth, appears to be amorphous, or with only a faint indication of further organization. This is separated from the main fibril-containing portion of the sheath by a narrow region exhibiting an oriented filamentous structure, the individual elements of which are *c.* 50 Å in width. Immediately beneath this occur the banded collagen-like elements of which longitudinal, transverse and oblique profiles are seen. The fibrils appear to range downwards from a diameter of *c.* 600 Å in a continuous size spectrum, and the

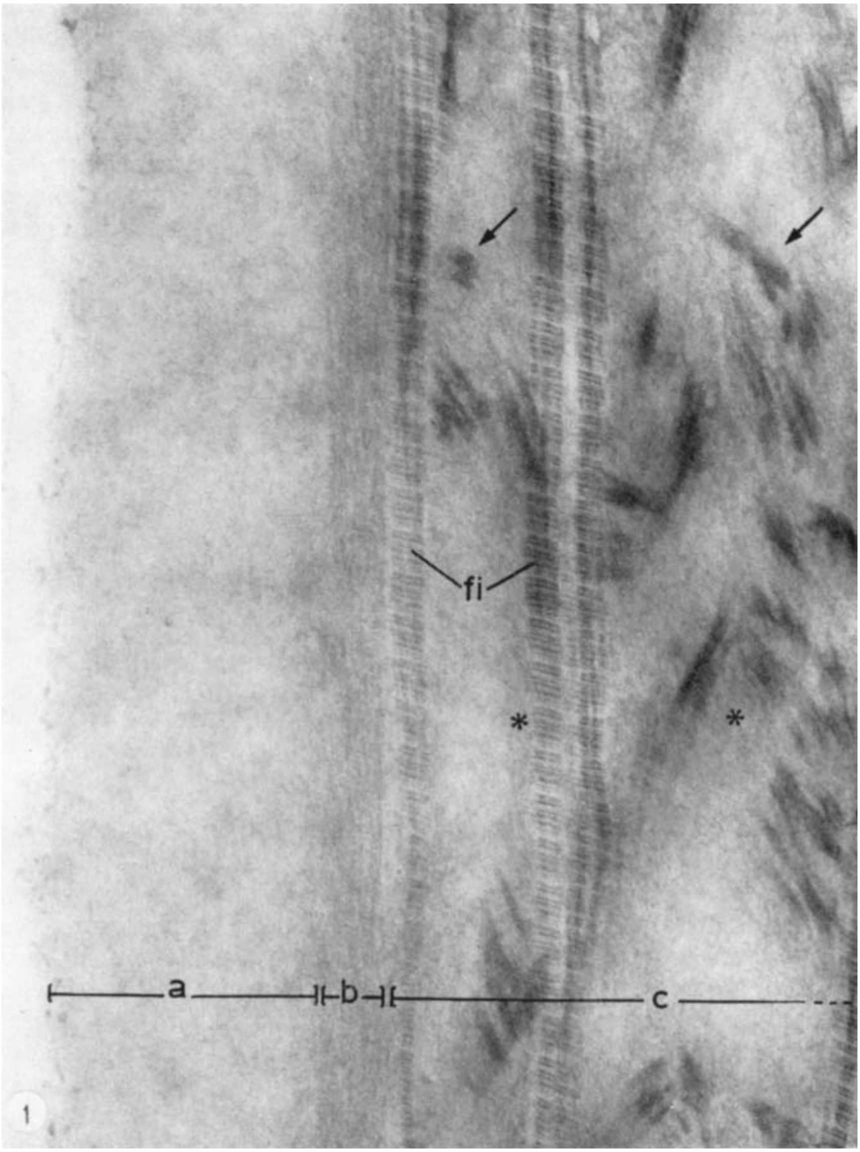

FIG. 1. Electron micrograph of a profile of the neural lamella or fibrous sheath of the last abdominal ganglion of *Periplaneta*. This field includes the surface and adjacent regions of the lamella. Note the outermost apparently amorphous layer (a) and the narrow underlying zone (b) containing fine filaments; also a portion of the main region of the neural lamella (c) containing fibrils of a collagen-like substance (fi). The banding of the latter is seen in longitudinal profiles and elsewhere (arrows) appear angular transverse or oblique fibril profiles. The amorphous matrix of the main portion of the sheath is of slightly higher density in the vicinity of the fibrils (*) than elsewhere. ×90,000.

characteristic periodicity has been noted in fibrils only 100–150 Å in diameter: there is, indeed, no evidence of the presence of non-banded fibrils in this portion of the lamella. In the field reproduced in Fig. 3, virtually all the fibrils are seen in longitudinal section, forming an interlacing open network, and apparently representing a horizontal profile of one of the "layers" of which the neural lamella is composed. The interfibrillar matrix of the lamella is apparently structureless, and except for zones of slightly higher density observed in the neighbourhood of the fibrils (Fig. 1), is as electron-transparent as the plastic embedding medium around the ganglion, in sections of osmium-fixed material that have been "stained" with lead or uranium salts.

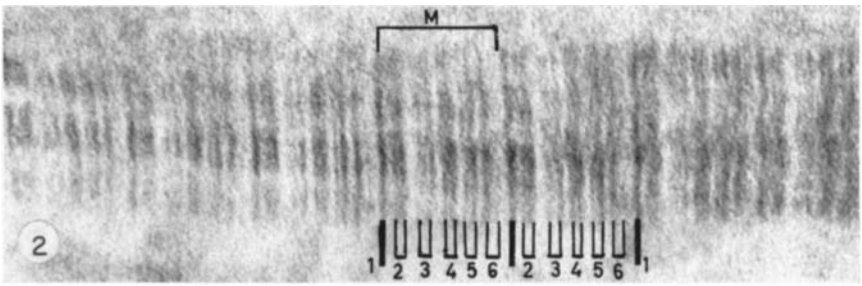

FIG. 2. Illustrating the band distribution in a collagen-like fibril of the neural lamella of *Periplaneta*. Each macroperiod (M) comprises a single dense subperiod line (1) and five apparently double subperiod lines (2, 3, 4, 5, 6). In this material the macroperiod is *c*. 560 Å in length. ×300,000.

There is, as yet, insufficient detailed information on which to base a comparison between the collagen-like fibrils observed in the neural lamella of different insects, or to relate these to the structurally well characterized "authentic" collagen of vertebrates. The macroperiod of fibrils observed in the unstained neural lamella of *Rhodnius prolixus* was found to be about 500–600 Å (Smith and Wigglesworth, 1959), while in sections the macroperiod in *Locusta* is 400–500 Å with "up to six interbands per period" (Ashhurst and Chapman, 1961); apparently corresponding to the pattern described by Baccetti (1961a) in *Aioloplus strepens*. In a comparative electron microscopic survey of the organization of the neural lamella in representatives of various orders, Baccetti (1961b) reported collagen-like fibrils, *c*. 550 Å thick with a macroperiod of 500–600 Å, in various *Orthoptera*; a similar period but thinner fibrils in *Thysanura*, *Ephemeroptera*, *Odonata*, *Hemiptera*, *Hymenoptera* and *Diptera*, although in *Coleoptera* and *Lepidoptera* Baccetti found an

indistinct periodicity, apparently of 150–200 Å. Gray (1959) made a study of the collagen-like component of the fibrous layer associated with the "fibrous sheath cell" and scolopale cell surrounding the dendritic portion of the auditory ganglion of *Locusta migratoria*, and took the important precaution (for purposes of comparison) of examin-

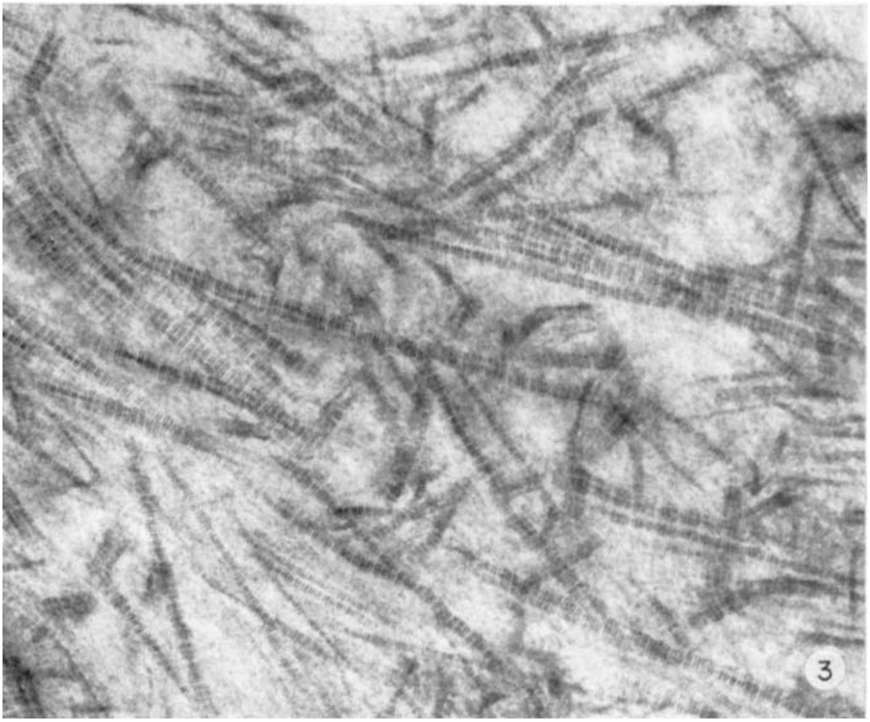

FIG. 3. A tangential section through the fibrous region of the neural lamella, in which most of the fibrils are seen in longitudinal section. The largest fibrils are *c*. 500–600 Å in diameter. Banding is detectable in the finest fibrils present, the diameter of which is *c*. 100–150 Å. ×50,000.

ing, together with the insect material, similarly prepared sections of rat-tail collagen. Gray found that, although the mean macroperiod of each was almost identical (*c*. 600 Å), the subperiod organization differed in detail, and he suggested that the deviation of the macroperiod in each sample from the generally accepted value for typical collagen of 640 Å, might be attributable to the mode of preparation and to the limitations inherent in magnification calibration of the electron

microscope. In this connection, it should be noted that whereas Ashhurst and Chapman measured a macroperiod of 400–500 Å in sections of *Locusta* neural lamella, they found that gold-palladium shadowed macerates of the ganglion contained fibrils with a periodicity of 600–650 Å.

The macroperiod interval in the fibrils of *Periplaneta* described by Treherne and Smith (in preparation) is approximately 560 Å; the

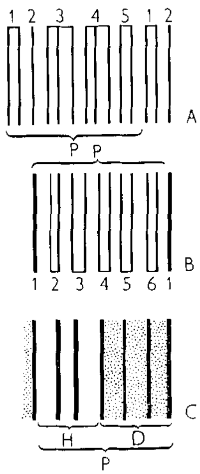

FIG. 4. Diagram illustrating interpretations of the subperiodicity occurring within the macroperiod (P) of the collagen-like fibrils of the insect neural lamella.

Gray (1959; A) considered the macroperiod in *Locusta* to consist of five subperiods, as indicated (1–5). In *Periplaneta* (B), Treherne and Smith (*in preparation*) found that the macroperiod appears to be defined by thick single subperiod bands (1) and five paired subperiod bands (2–6). This diagram illustrates the possible equivalence of these two interpretations. Similarly, it is possible that the model provided by Baccetti (1961a; C) for the fibrils in *Gryllus* may relate to the same material: except for the doubling of the subperiod bands in *Periplaneta*, the interpretations shown in B and C correspond closely.

Note that the subperiod bands in this diagram have not been drawn accurately to scale, and that the interband intervals are in reality very narrow (cf. Fig. 2). Note also that while the macroperiod has here been indicated as identical in each instance, the variability of this value described in different accounts (500–600 Å) may, as Gray (1959) suggests, reflect an artefact introduced during specimen preparation.

banding consisting of single, very dense lines, taken as defining the macroperiod, separated by ten subperiod lines of varying density (Fig. 2). The latter are associated into five doublets. At first sight this pattern does not seem to correspond to the periodicities reported by Baccetti and Gray, but a closer comparison indicates that all these accounts probably relate to a similar material, as is shown in Fig. 4, in which the various inter-

pretations are compared directly. It thus appears that the "collagen" of *Periplaneta*, and the other species examined, resembles that of the rat-tail illustrated by Gray, except for the insertion of an extra doubled subperiod band between the single bands defining the limits of the macroperiod as described by Schmitt *et al.* (1955).

Baccetti (1961a) noted that the neural lamella of *Blaps gibba* contains appreciable amounts of hydroxyproline, and gives a diffraction pattern indicative of the presence of collagen. Only very thin fibrils, 100–200 Å in diameter, with a period of 150–200 Å, were seen in the electron microscope. Ashhurst and Chapman (1961) also found a few unbanded fibrils of similar size occurring along with the large banded components in the macerated neural lamella of *Locusta*. It should be remembered, however, that it is considerably easier to detect and measure periodic banding in thick rather than thin fibrils, so this anomaly may be apparent rather than real. Of greater interest is Baccetti's report (1961a) that the stroma (basement membrane) around the alimentary canal of *Ailopus* and *Blaps* contains fine fibrils in which no periodicity was detected, and in this instance no indication of the presence of collagen-like material was found by other methods: no appreciable amounts of hydroxyproline were present, and X-ray diffraction bands indicated denatured protein only. It is possible that, in insects, collagen may prove to be developed specifically in association with the nervous system, and this may be restricted to the central nerve chain, since although banded fibrils occur in the neural lamella around the ganglia and connectives, the extracellular layer surrounding the nerve sheath in the peripheral nerves is very thin, apparently devoid of fibrils, and corresponds, in its appearance in electron micrographs, to a typical basement membrane.

Ashhurst (1959) reviews the evidence for the occurrence of neutral polysaccharide in the neural lamella, which may either be chemically linked or less intimately associated with the collagen fibrils. She points out that Bradfield (1950) suggested that the plasticity of connective tissue fibres is related to the amount of polysaccharide present, and concludes that if the insect neural lamella is rich in this material, then "this would seem to agree with the mechanical functions of the neural lamella, which are to hold together the cells and axons of the nervous system and yet to be flexible enough not to resist or impede the movements of the body". In addition, it is possible that the arrangement of the collagen fibrils within the neural lamella renders it relatively inextensible. The neural lamella may thus be mechanically equipped to resist the positive hydrostatic pressure resulting from the osmotic

pressure excess produced in the extracellular fluid by the Donnan equilibrium with the haemolymph (Treherne, 1962a, b). The almost explosive bursting-out of nerve substance through small holes made in the nerve sheath, described by Twarog and Roeder (1956), is convincing evidence of the mechanical resistance to swelling which this membrane affords.

The neural lamella appears to offer little resistance to diffusion of the various substances which have been tested. Twarog and Roeder (1956) found that silver nitrate penetrated this fibrous layer in the cockroach ganglion, while Wigglesworth (1960a) describes this structure as being apparently freely permeable to dye molecules. The relative permeability of the neural lamella in *P. americana* can also be inferred from the rapid movements of ions such as ^{24}Na and ^{42}K between the haemolymph and the abdominal nerve cord (Treherne, 1961a) and from the ability of ^{14}C-labelled inulin molecules to penetrate into the extracellular spaces of the central nervous system (Treherne, 1961e, 1962b).

The considerable collagen content of the neural lamella may influence the ionic composition of the fluid contained in this structure. According to Tristram (1953) collagen contains 77·2 free anion equivalents/10^5g protein. The possibility exists, therefore, that there may be an appreciable proportion of the free anionic groups capable of association with inorganic cations in the neural lamella. Thus the ionic composition of the fluid in contact with the cells of the perineurium may well be very different from that of the haemolymph. The mucopolysaccharide ground substance described by Ashhurst (1959) is apparently neutral in reaction, and unlike the acid mucopolysaccharide component of the extracellular spaces (Ashhurst, 1961c; Treherne, 1962b), is unlikely to influence the ionic content of the neural lamella.

B. THE PERINEURIUM

The perineurium, according to the usage adopted here, constitutes the cellular layer immediately underlying the neural lamella of the ganglion and lying externally to the glial cells associated with the cell bodies of the neurones. With the neural lamella these cells form the sheath of the ganglion.

The importance of these cells in the metabolism of the nervous system has been stressed by Wigglesworth (1959b; 1960a). He pointed out that the insect nervous system is an avascular organ and that apart from the oxygen carried by tracheae and tracheoles, all the necessary exchange of nutrient and excretory substances must take place through the perineurium. Histological studies on these cells have been made by

Scharrer (1939) in *Periplaneta*, by Wigglesworth (1958, 1960a) in *Rhodnius* and *Periplaneta* and by Ashhurst (1961b) in *Periplaneta*, while the cytological organization of the perineurium in *Periplaneta* has been described by Hess (1958a) and Treherne and Smith (in preparation).

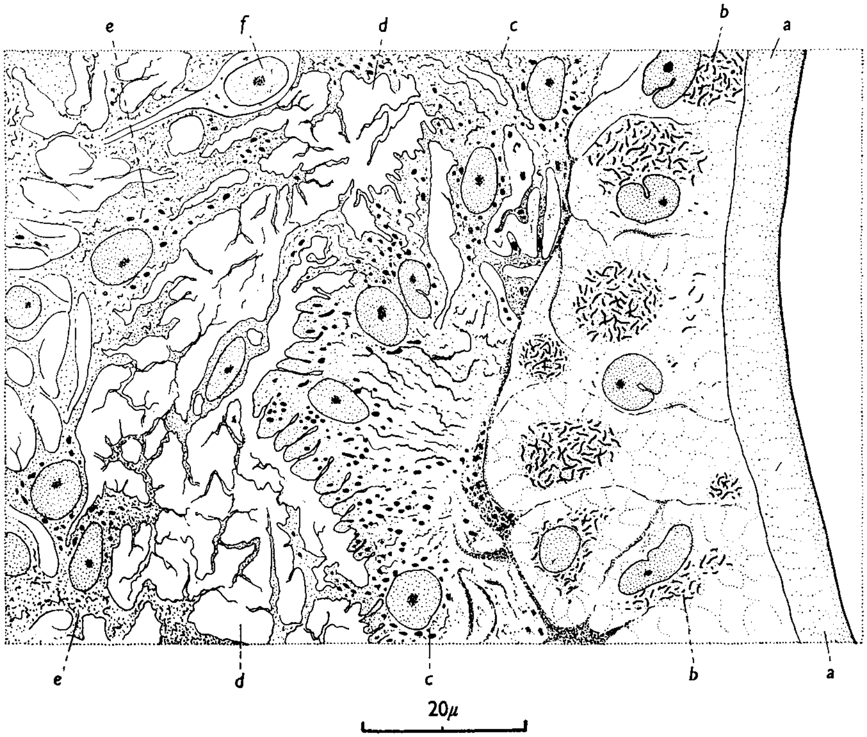

FIG. 5. The peripheral region at the anterior end of the terminal abdominal ganglion of *Periplaneta americana* just dorsal to the connectives. (a) neural lamella; (b) perineurium cells with clumps of filamentous mitochondria; (c) outer glial cells with oval and rod-like mitochondria; (d) glial sinus system, partially collapsed; (e) inner glial cells bounding the neuropile; (f) small neurone (Wigglesworth, 1960a).

Large quantities of glycogen occur within the cytoplasm of the perineurium cells (Wigglesworth, 1960a; Ashhurst 1961b, 1959) and while Ashhurst found numerous "lipochondria" in this region, believed to contain phospholipids and cerebrosides, Wigglesworth (1960a) noted that triglyceride droplets are only occasionally seen in the perineurium of *Periplaneta* in osmium-ethyl gallate preparations. All investigations have shown abundant mitochondria in the perineurium, and Ashhurst

reported strong basiphilia in the cytoplasm which was reduced after treatment with RNA-ase.

Wigglesworth (1960a) has described the topography of the perineurium in *Periplaneta* (Fig. 5); this forms a narrow epithelium laterally, and is hypertrophied anteriorly, near the origin of the connectives linking the last abdominal ganglion with its neighbour, and also posteriorly, near the insertion of the cercal nerves. The cytological organization of these cells has been investigated by Hess (1958a), Trujillo-Cenóz (1962) and by Treherne and Smith (in preparation), and the main features are illustrated in Figs. 6, 9–11. These cells do not have a conspicuous basement membrane (Fig. 6); the outer plasma membrane is overlaid, usually without interruption, by the basal region of the neural lamella. Hess described the distribution of bodies with the characteristic internal structure of mitochondria: elongate and circular profiles of these abound on the cytoplasm and the well separated cristae of each mitochondrion lie in a dense homogeneous matrix (Fig. 11). As Wigglesworth noted, the mitochondria often have an aggregated distribution in the cells; in electron micrographs they are often found in large number immediately adjoining the neural lamellar surface (Fig. 10) and in clusters deeper in the cytoplasm.

The presence of the cellular epithelium, together with the overlying fibrous sheath, has been found to exert a profound effect on the electrical behaviour of the underlying conducting elements, especially in conditions of abnormal composition in the haemolymph or bathing solution. This effect was first demonstrated in the peripheral nerve of the locust, when it was shown that, with solutions containing relatively high concentrations of potassium ions, removal of the sheath or injection beneath it resulted in depolarization which occurred more rapidly than in most intact preparations (Hoyle, 1953). Essentially similar results were obtained on desheathing portions of the cockroach abdominal nerve cord in the presence of abnormally high concentrations of

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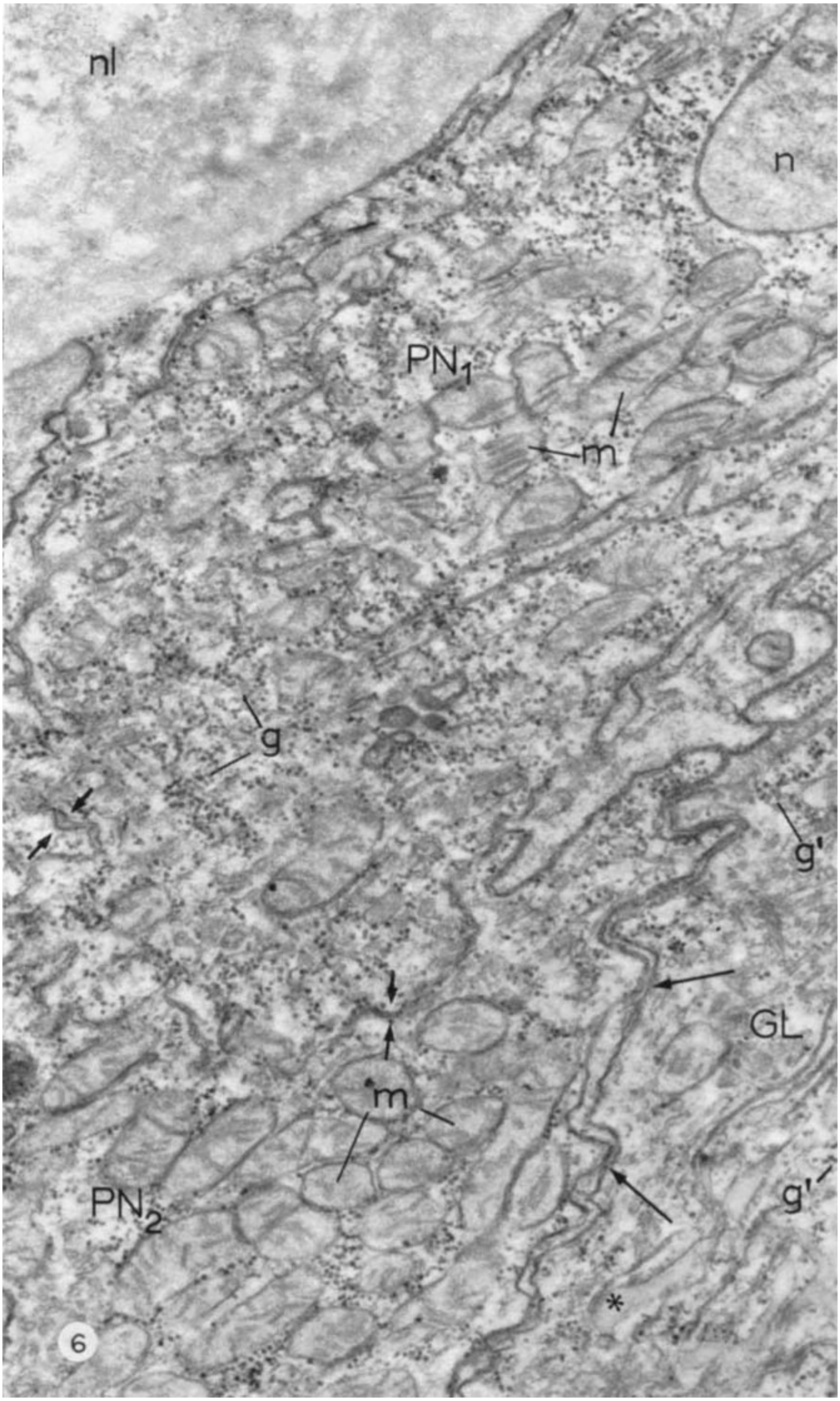
FIG. 6. Illustrating the spatial relationship between the neural lamella (nl), perineurium and underlying glial cytoplasm in *Periplaneta*. Portions of two perineurium cells are present (PN_1), (PN_2), the sinuous apposed cell membranes of which are indicated by short arrows. At the right of the field, the perineurium cells abut onto a glial cell (GL) as at the points indicated by long arrows. The cytoplasm of the perineurium cells contains large numbers of mitochondria (m) and much of the intervening space is filled with deeply "stained" granules (g) *c*. 300–350 Å in diameter, believed to represent glycogen. Smaller numbers of similar granules also occur in the cytoplasm of the glial cell (g′) (cf. Fig. 15). At extreme lower right are included profiles of glial cell folds, between the membranes of which occurs an extracellular amorphous material (*) seen more clearly in Figs. 7, 13, 15, etc. A portion of a nucleus is included at (n). × 29,000.


nl
n
PN1
m
g
g'
GL
m
PN2
g'
*
6

potassium ions or acetylcholine molecules (Twarog and Roeder, 1956). The sheath enveloping the locust nerve appeared to be more effective in resisting the adverse effects of high concentrations of potassium ions than that of the cockroach central nervous system. With concentrations of up to 70 mM K^+ the locust axons exhibited little change of potential for periods of over 6 h (Hoyle, 1953); solutions of similar concentrations applied to the cockroach abdominal nerve cord blocked conduction in only a fraction of this time (Fig. 7) (Twarog and Roeder, 1956; Treherne, 1962c).

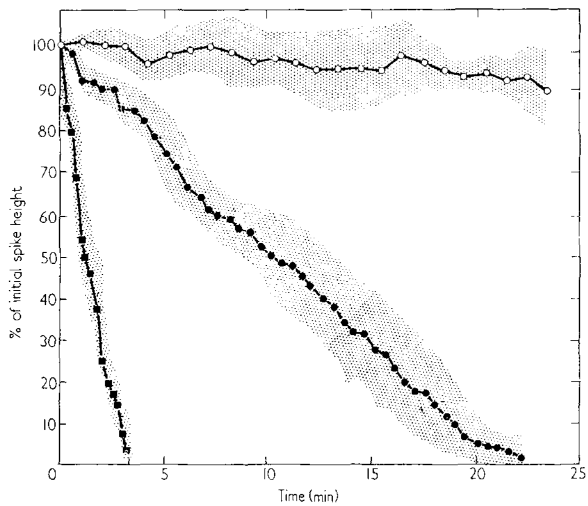


FIG. 7. The rate of loss of conduction through the fourth abdominal ganglion of *Periplaneta americana* when irrigated with normal physiological solution (open circles) and with a high potassium solution (70 mM/l K^+) (closed circles). The desheathed ganglion (closed squares) was irrigated with the high potassium solution. The shaded areas represent the extent of twice the standard error of the mean (Treherne, 1962c).

In view of the apparent permeability of the neural lamella these results have, in general, been interpreted as demonstrating the function of the perineurium as a significant diffusion barrier restricting the entry of ions and molecules into the underlying nervous tissues. In their study of the fine structure of the peripheral nerves in the wasp Edwards *et al.* (1958a), for example, suggested that while the neutral lamella might limit the rate of flow of ions, the sheath cells would actually form the "selective ion barrier". The relatively rapid movements of ^{24}Na and ^{42}K between the haemolymph and the nerve cord of *P. americana* clearly

showed that a dynamic steady state rather than a static impermeability must occur across the nerve sheath in the cockroach (Treherne, 1961a). Sodium ions were found to be actively extruded from the nerve cord in this insect and it was assumed that this steady state was at least partially effected by a metabolically maintained linked-sodium pump (Treherne, 1961b). Such an extrusion might have been regarded as evidence for a selective role played by the sheath cells as postulated by

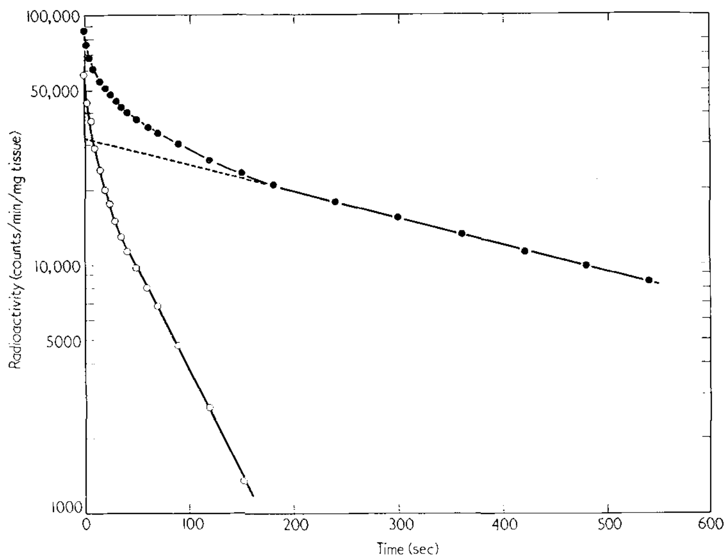


FIG. 8. The escape of ^{24}Na from an isolated abdominal nerve cord of *Periplaneta americana* when washed in inactive physiological solution (closed circles). The fast component (open circles), which has been identified with the extracellular ion fraction, was obtained by subtraction from the straight line extrapolated to zero time. The slow exponential decline in radioactivity, which was reduced in the presence of metabolic inhibitors, has been identified with the cellular ion fraction (Treherne, 1961e).

Edwards and his co-workers. Subsequent investigation showed, however, that the metabolically maintained effluxes of sodium ions were not those taking place across the fibrous and cellular nerve sheath, but were probably the ion transfers associated with the underlying cells of the central nervous system (Fig. 8) (Treherne, 1961c, d). The movements of mono- and divalent ions were, in fact, found to take place extremely rapidly across the perineurium, in their exchanges between the haemolymph and the extracellular fluid in the abdominal nerve cord in the cockroach (Treherne, 1961e, 1962b). No evidence was

obtained for any active process involved in the movements of the ions between the haemolymph and the extracellular spaces.

The relatively rapid movement of ions and molecules across the perineurium in the cockroach is difficult to reconcile with the concept based on electrophysiological evidence, of the nerve sheath as a diffusion barrier restricting entry into the underlying tissues. An alternative hypothesis which has been advanced will be considered in a subsequent section dealing with the disposition of the extracellular spaces of the central nervous system. It should be pointed out that this latter hypothesis does not necessarily extend to peripheral nerves of such species as the locust, which have been shown to function differently from the central nervous system of the cockroach in solutions containing excessive amounts of potassium ions (Hoyle, 1953).

The most striking cytological feature of the perineurium cells is the presence of very great numbers of deeply "staining" granules *c.* 300–350 Å in diameter (Treherne and Smith, in preparation). These may sometimes form aggregates filling large areas of the cell (Figs. 10 and 11), and while groups of mitchondria are occasionally found not to be associated with the granules (Fig. 9), these two cell components are usually freely intermingled (Fig. 6). Profiles of smooth membraned vesicles and cisternae (agranular endoplasmic reticulum) are scattered throughout the cytoplasm, and these sometimes form parallel arrays or Golgi bodies in the region of the nucleus. Particle-bearing cisternae appear to be sparsely distributed throughout the cell. The nuclei of the perineurium cells are sometimes seen as ovoid profiles, but the nuclear envelope may be deeply indented (Fig. 9).

Wigglesworth (1960a) and Treherne (1960) have stressed the rôle

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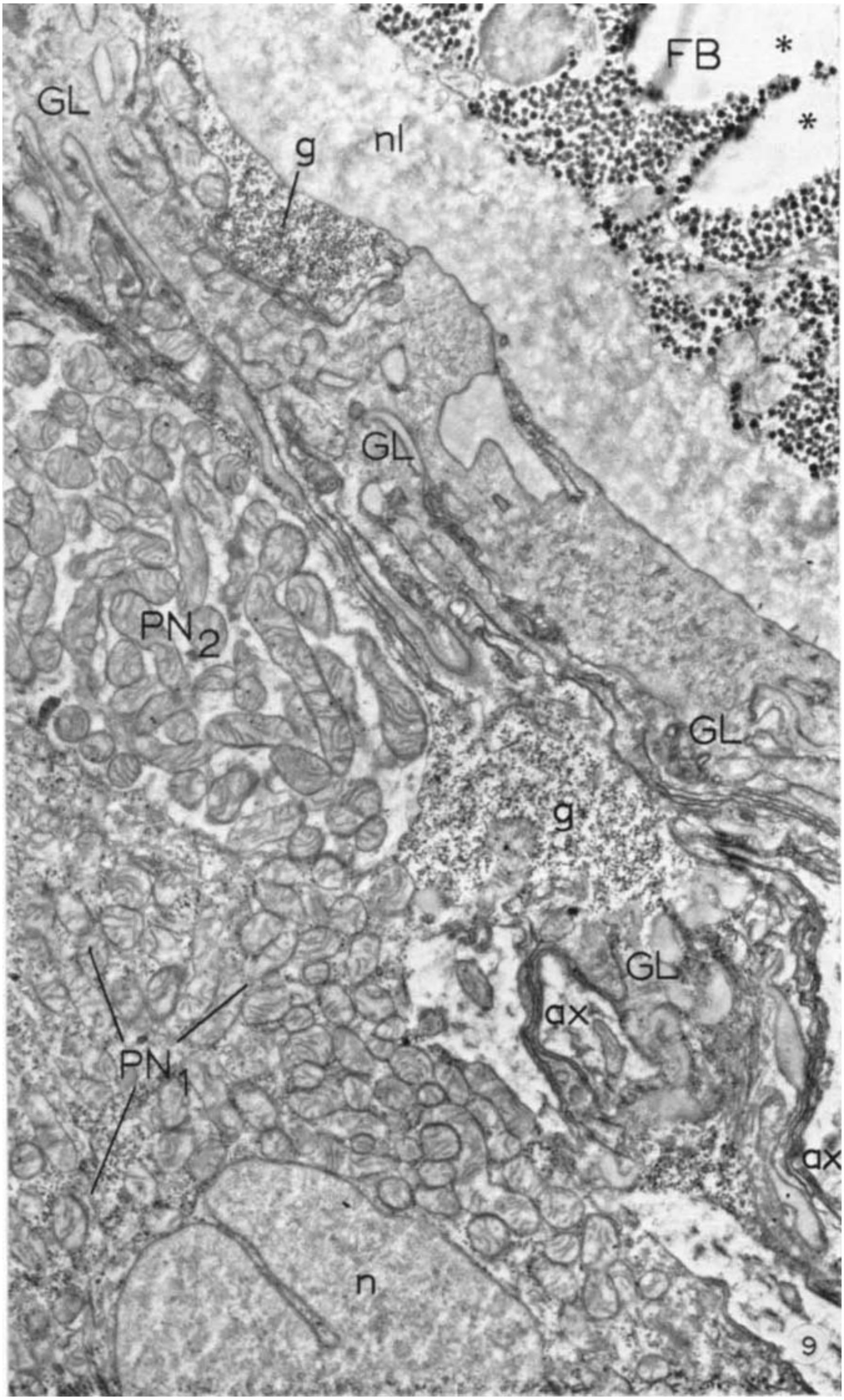
FIG. 9. Longitudinal section of *Periplaneta* ganglion close to the origin of a connective. A portion of a fat-body cell (FB) containing clusters of deeply "stained" glycogen deposits, and large cavities (*). The latter probably represent the location of fat droplets lost during preparation of the material, nl indicates the neural lamella. The bulk of this field is occupied by perineurium cytoplasm: the nucleus of one cell (PN_1) is seen at n, and the surrounding cytoplasm is filled with mitochondria and glycogen granules. At PN_2 appears a portion of another perineurium cell containing a mass of mitochondria not associated with granular deposits of glycogen which are tightly packed elsewhere (g) in the perineurium cells—a feature illustrated in Figs. 10 and 11.

A glial cell process (GL) with dense cytoplasm, of the cellular layer underlying the neural lamella in the connectives (cf. Fig. 12), traverses the field diagonally, and is seen in oblique section at lower right where profiles of two axons (ax) are associated with it. Granules believed to represent glycogen, similar in appearance to those found in the perineurium cells, occur in the glial cytoplasm, notably at the base of the cercal nerves and connectives: these are present in the vicinity of the axons in this figure, and are seen to better advantage in Fig. 15. ×13,000.


FB
*
*
GL
g
nl
GL
PN2
GL
g
GL
ax
PN1
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n
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played by the perineurium in the passage of nutrients into the ganglion, and have studied biochemical and histochemical aspects of carbohydrate mobilization in *Periplaneta*. Wigglesworth found that glycogen

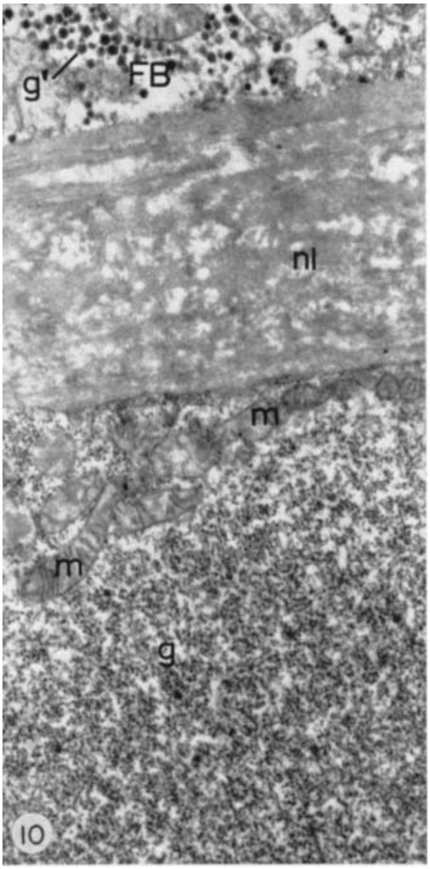

FIG. 10. A peripheral field in the ganglion of *Periplaneta*, showing a small portion of fat-body cytoplasm (FB), the neural lamella (nl) and the margin of a perineurium cell. The latter is filled, in this region, with granules of glycogen (g), also occurring in clusters (g') in the fat-body cell (cf. Fig. 12). Note the mitochondria (m) within the perineurium cell, close to the base of the neural lamella. × 16,000.

is abundant both in the fat body surrounding the abdominal ganglion and also in the perineurium, while smaller amounts of glycogen were found in the glial processes investing the neurones and axons in the ganglion and in the circular sheath around the axons in the cercal

nerves and abdominal connectives. Wigglesworth's observation that the inner glial cells act as a morphological and physiological link between the perineurium and nerve components will be discussed in due course; we are here concerned with the "peripheral" aspect of

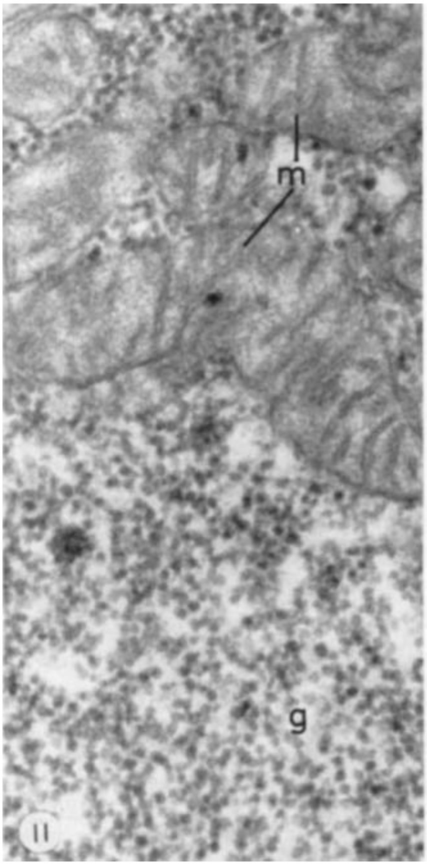

FIG. 11. A field similar to that illustrated in Fig. 10, at higher magnification. Note the glycogen granules (g) *c*. 300–350 Å in diameter, and the mitochondria (m) containing subparallel cristae. $\times 60,000$.

nutrient mobilization—the relationship between the perineurium, the neural lamella and extraganglionic materials. In addition to being exposed to the surrounding haemolymph, the neural lamella is very intimately associated with the fat-body cells (Treherne and Smith, in preparation). The latter have a very characteristic cytological structure

as seen in electron micrographs (Figs. 9, 10, 12); large membrane-limited cavities are present, probably representing the sites of fat droplets lost during preparation, though some osmiophilic bodies are retained. Subspherical or elongated mitochondria, larger and with a more extensive matrix than those within the perineurium, are distributed throughout the cell. The most conspicuous feature, however, is the presence within the cytoplasm of large numbers of clusters of deeply "stained" granules, often filling the bulk of the cell. The individual granules are similar in size and appearance to those occurring in the perineurium cells, the clusters being of various sizes, often approximately 0·1 μ in diameter. Revel *et al.* (1960) identified similar granules as glycogen in muscle of the frog tongue and turtle heart, in the chick glycogen body and in brown fat cells of foetal mice. The granules in the fat body and perineurium of *Periplaneta* fall within the size range (150–400 Å) reported by Revel and his co-workers, while the appearance of the granules in lead-stained material from each source is similar.

Of particular interest here is the possible relationship between the granules believed to represent glycogen in the fat body and those packing the perineurium cells on the inner side of the neural lamella. Wigglesworth (1960a) found that in cockroaches starved for 3 to 4 weeks glycogen is absent from the fat body, and, except for minute deposits in the perineurium, has also disappeared from the ganglion. Glycogen was found to be rapidly replaced within the ganglion, and notably within the perineurium, upon feeding the insect with sugars. Similarly, a synthesis of glycogen within the central nervous system of mosquito larvae was demonstrated by following its accumulation upon feeding starved individuals with carbohydrates and amino acids (Wigglesworth, 1942). It has also been shown that injected ^{14}C-glucose is rapidly converted to the disaccharide trehalose which accumulates in the haemolymph, the radioactivity of which quickly appears among a variety of compounds,

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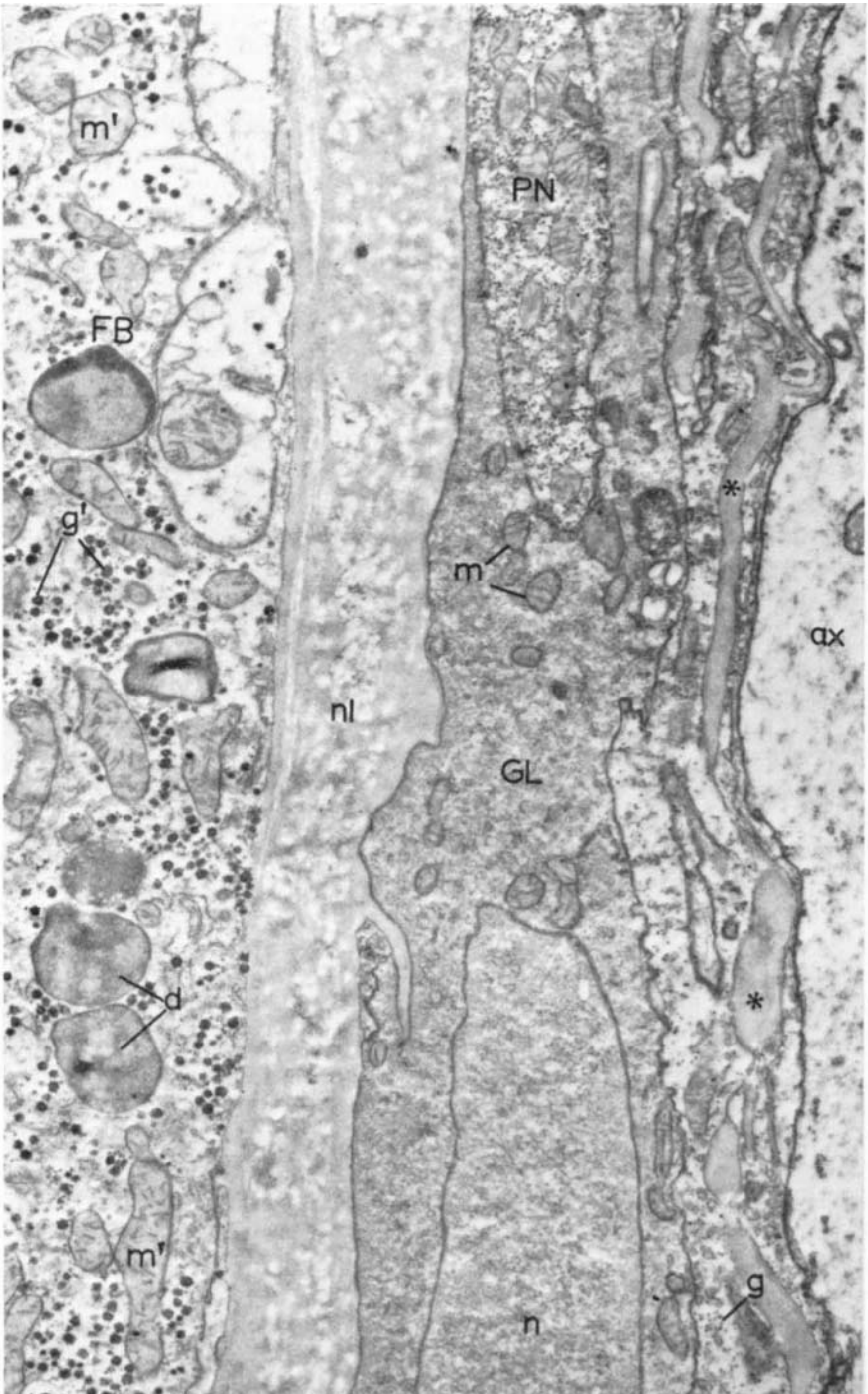
FIG. 12. A longitudinal profile of the peripheral region of an inter-ganglion connective, close to its origin in the last abdominal ganglion. The neural lamella (nl) is here only *c.* 1·0–1·5 μ in thickness. This is flanked externally (on left) by fat-body cytoplasm (FB) containing glycogen granules (g′), large mitochondria containing few cristae (m′) and osmiophilic droplets (d) probably of lipid material. A glial cell (GL) lies beneath the neural lamella: a portion of the nucleus of this cell is included at n, and the surrounding cytoplasm is dense and finely granular and contains sparsely distributed mitochondria (m). The glial processes surrounding the axon (ax) at right contain a few glycogen granules (g), and the extracellular spaces between these processes contain an amorphous material (*).

At PN is seen a cytoplasmic profile containing clusters of mitochondria and glycogen deposits; this may represent a perineurial prolongation extending into the connective. ×16,000.


m'
PN
FB
g'
m
ax
nl
GL
d
m'
g
n
12

including glycogen, in the tissues of the cockroach abdominal nerve cord (Treherne, 1960). It is evident, then, that despite the postulated impermeability of the nerve sheath, these various nutrient substances are readily made available to the synthetic mechanism of the cellular components of the ganglion.

In the light of the apparent permeability of the neural lamella to ions and molecules, which has already been commented upon, it is possible that the intimate relationship between the fat body and the nerve sheath may indicate that the metabolic processes of these cells are closely linked across the fibrous layer. It is possible, for example, that the granules seen in electron micrographs of the perineurium represent glycogen synthesized in these cells from metabolic intermediates (some of which may be degraded from fat-body glycogen) that have diffused across the neural lamella and perineurium plasma membranes from the adjacent fat-body cells. The abundance of mitochondria in both the fat body and the perineurium cells may be correlated with this synthetic function: Treherne (1960) showed that a substantial part of the tricarboxylic acid cycle enzymatic activity occurs in the abdominal nerve cord of *P. americana* and these enzymes are generally recognized to be associated with mitochondria in other cells.

In addition to effecting the exchanges of the necessary nutrient and excretory substances, the perineurium must also be specialized to allow the passage of various neurosecretory products into the haemolymph. The neurosecretory cells of insects are modified neurones and are invariably situated beneath the perineurium, so that the release of the secretory products into the haemolymph involves penetration of both the fibrous and cellular layers of the nerve sheath. In *Rhodnius* the diuretic hormone produced in the fused meso- and metathoracic and abdominal ganglia appears to be released rapidly from this structure, since 2·5 min after ingestion of a blood meal the malpighian tubes are stimulated to maximum secretion (Maddrell, 1962a, b). In this case it seems reasonable to suppose that the actual passage of the hormone across the perineurium takes place at a rate which occupies only a fraction of this time. These observations can be readily accounted for according to the hypothesis of a perineurium which is passively permeable to ions and molecules up to and probably greater than the size of inulin. According to any theory of rigid selective permeability, on the other hand, it would be necessary to postulate active secretory processes for each of the various neurosecretory products released into the haemolymph.

Wigglesworth (1959b) suggested that the perineurium, although

forming a cellular sheath distinct from the underlying non-nervous elements investing the nerve cells, should be regarded as consituting part of the neuroglial system: that the single type of sheath cell present in peripheral nerves ("Schwann cell", "lemnoblast") becomes differentiated within the central ganglia into the perineurium, and into other specialized cells that act as intermediaries for the passage of materials to and from the neurone cell bodies, and also contribute the inter-axon component in the central neuropile. However, although it is evident from the work of Wigglesworth that these cells are functionally linked in the metabolism of the ganglion, there is, as Pipa (1961) points out, no clear information on the embryological derivation of the perineurium and glial cells. Moreover, the rôle (if any) played by the perineurium in the secretion of the neural lamella has not yet been demonstrated: Ashhurst (1959) detected no alkaline phosphatase activity in these cells (suggested by Bradfield, 1946, to indicate sites of fibrous protein elaboration in insects), but it is possible that the perineurium plays a dual part during the development of the ganglion; first, in secreting a fibrous connective tissue sheath, and later in functioning as a trophic epithelium.

III. Glial Cells

Wigglesworth (1960a) observed that in the ganglia of *Periplaneta* the subperineurial glial system is separated into two rather poorly defined regions: a peripheral cell layer investing the neurone cell bodies, and an inner layer lying at the surface of, and sending a complex system of processes into the neuropile. These two layers and the cells comprising each were found to be partially separated by an irregular system of sinuses, the "glial lacunar system" (Figs. 13, 14), the extent of which varies greatly depending on the nutritional state of the insect, being most extensive in old or starved individuals.

It is evident, even from the limited information at present available, that variation exists between different insects in the distribution and degree of differentiation shown by the neuroglial cells lying beneath the perineurium. In general these cells form two main groups within the ganglion: those lying immediately beneath the perineurium invest the neurone cell bodies, while a deeper layer defines the outlines of, and sends extensively arborizing cytoplasmic processes into the neuropile, where they are associated with the axons and their branches. In *Rhodnius* certain glial cells form a thick "myelin-like" sheath around the large motor axons as they leave the ganglion, while specialized cells characterized by giant nuclei surround the nerve cell bodies, and further

differentiation of the neuroglia occurs in the brain (Wigglesworth, 1959b). "Giant" glial cells have been found in representatives of several orders (see Pipa, 1961, for refs.), but do not occur in *Periplaneta* and other roaches (Wigglesworth, 1960a; Pipa, 1961). The trophic function of the neuroglia in supplying glycogen and lipids to the neurones has been demonstrated by Wigglesworth (1960a) and Treherne (1960): the part

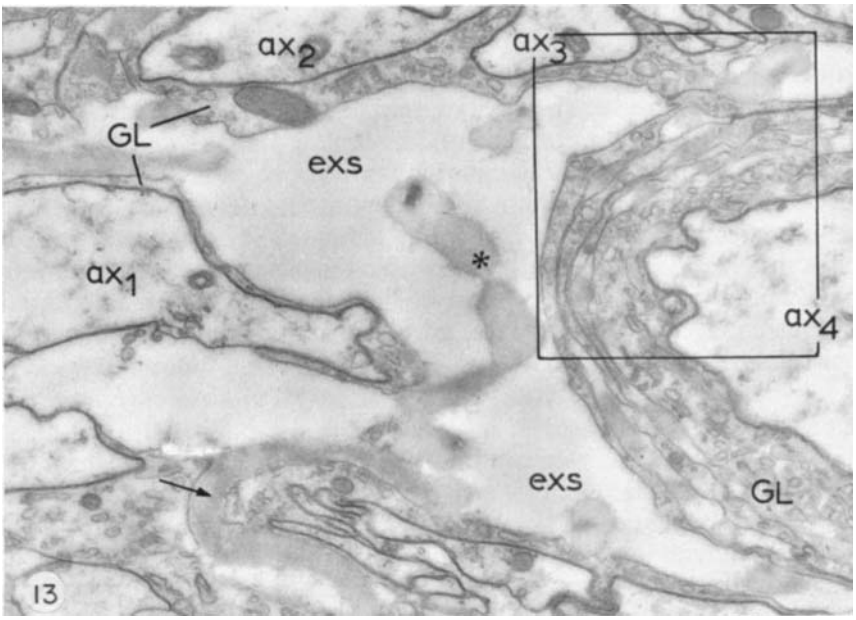


FIG. 13. Illustrating extensive extracellular spaces (exs) between the glial cells in the ganglion of *Periplaneta*; presumably representing the "glial lacunar system" of Wigglesworth (1960a). The axon at right (ax_4) is invested with a thick glial sheath (GL) of concentric turns of the invaginated mesaxon (cf. Fig. 14), while the axons at ax_{1-3} have only a very thin glial covering. Note the amorphous material present in this field; this may follow the outline of the glial surface (arrow) or extend across the extracellular space (*). × 17,000.

played by the perineurium in the elaboration and storage of the glycogen has been discussed previously.

Wigglesworth (1960a) found that after diastase digestion, PAS-positive material remained beneath the ganglion sheath, as "thickenings in the membranes around the glial cells and axons" (appearing as a network around the latter, in surface section, cf. Pipa and Cook, 1958), as a lining of, and as filaments crossing the glial lacunar system, and as irregular deposits and "membranes" elsewhere in the neuroglia. Hess

(1958a) and Pipa (1961) demonstrated an acid mucopolysaccharide material occurring between the inner and outer glial layers: Hess believed this to be within the glial cytoplasm, and Pipa was not able to

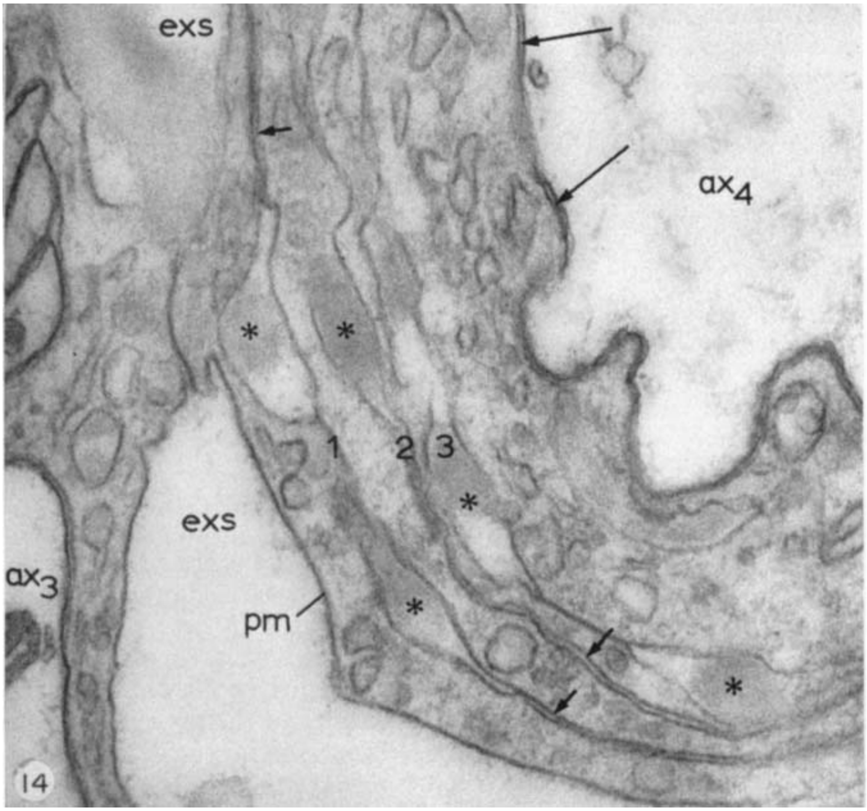


FIG. 14. The "boxed" portion of the field shown in Fig. 13, at higher magnification. The superficial plasma membrane of the glial sheath of axon ax_4 is seen at pm, and the sheath in this region comprises three mesaxon turns (1, 2, 3), the apposed membranes of which are separated by an extracellular gap of *c.* 100 Å for part of their course (short arrows), but at intervals form local dilatations 100–200 m. wide, often containing a dense amorphous material (*). The innermost glial membrane surface is separated from the plasma membrane of the axon by a gap of *c.* 100 Å (long arrows). The extensive extracellular space between the glial processes is seen at exs. ×50,000.

distinguish its precise location; whether extra- or intracellular. Ashhurst (1961c) described an extracellular region between the glial cells, containing histochemically similar PAS-negative material, present in several

genera of roaches (*Periplaneta*, *Blaberus*, *Blatta*, *Blattella* and *Diploptera*), but apparently not found in *Locusta* (Ashhurst), *Ephestia* (Richards, quoted by Ashhurst), sucking lice (Pipa and Cook, 1958) or *Oncopeltus* (Pipa, 1961). Small glial cytoplasmic inclusions, believed to be distinct from mitochondria, were described as "gliosomes" by Scharrer (1939), and Pipa (1961) found that they are PAS-positive in diastase-digested material and appear to contain glyco- or phospholipid associated with protein: these were not observed, however, in *Oncopeltus* (Johansson, 1957) or *Rhodnius* (Wigglesworth, 1959b). Pipa *et al.* (1962) have examined the structure of gliosomes of *Periplaneta* in the electron microscope. These bodies were found to occur in the glial cells surrounding the perikarya, in the sheaths of the peripheral nerves and, most abundantly, in the glial cell layer bounding the neuropile. They are revealed as irregular membrane-limited structures, 0·5–4·0 μ in length, containing an osmiophilic granular matrix and often arrays of myelin-like membranes. These investigators pointed out that it is not yet clear whether the bodies described as "gliosomes" in different species are homologous structures, but in *Periplaneta* they evidently constitute a distinct cytoplasmic component of the gliocytes.

The main features of the cytological organization of the glial cells of *Periplaneta*, and of their relationship to the perikarya and axon processes of the neurones, has been described by Hess (1958a) and Treherne and Smith (in preparation), and some indications of the possible structural identity of the histochemically recognized components described above have been obtained. Hess found that the nucleus of the glial cell, whether from the inner or the outer layer, is surrounded by a narrow zone of cytoplasm, and the remainder of the cell is dissected in a very complex fashion: the inner glial cells send cytoplasmic processes into the neuropile, while those of the outer layer form sheet- or ribbon-like arrays around the neurones, often, as Hess observed, in multiple or concentric fashion, giving these cells a "fibrous" appearance in the light microscope. The glial cytoplasm is quite distinct from that of the perineurial cells: the mitochondria are larger and fewer in number, and the perinuclear and peripheral regions of the cell contain small profiles of tubular elements of the granular (RNP-particle bearing) and agranular endoplasmic reticulum. Deeply "stained" granules, identical with those present in the perineurium cells, are often seen in the glial cytoplasm. These are most evident in the glial processes associated with the axons at the base of the connectives (Figs. 9, 15) and cercal nerves, and the supposition that these represent glycogen is strengthened by Wigglesworth's observation (1960a) that

histochemically demonstrated glial glycogen is most evident in these regions of the ganglion.

The intimate relations between gliocyte processes and the surface of the neurone cell body is well established; in light microscopic preparations the surface of the latter is seen to be deeply and irregularly invaginated to accommodate narrow processes of the glial envelope

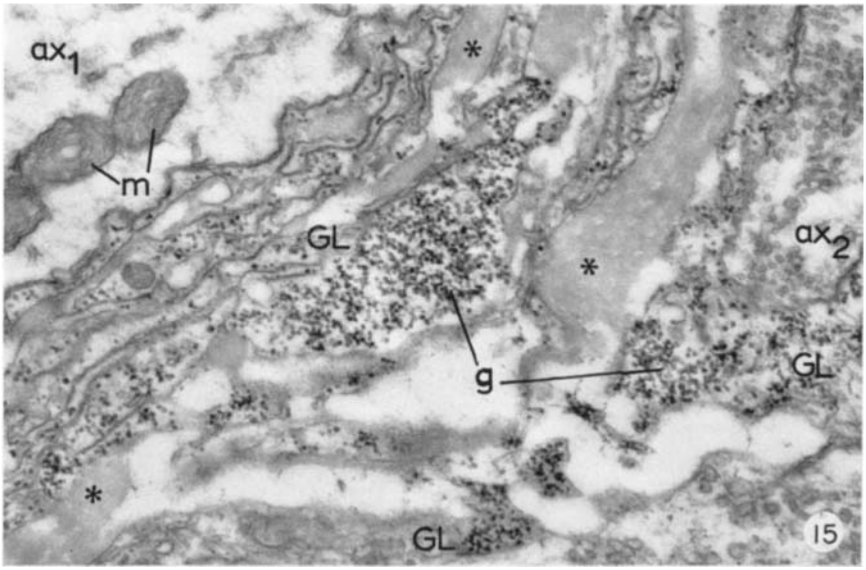


FIG. 15. Illustrating a region at the junction between the last abdominal ganglion of *Periplaneta* and a connective. Two axon profiles are present (ax_1, ax_2): the former contains mitochondria (m), and the latter, clusters of vesicles which appear to be concentrated in the axoplasm as it approaches termination. The glial cell processes (GL) associated with the axons contain large numbers of granules (g) similar to those found in the perineurium (cf. Figs. 6, 9, 10, 11) and believed to be of glycogen. A substantial portion of the extracellular space lying between the glial prolongations is filled with a dense amorphous material (*). $\times$ 25,000.

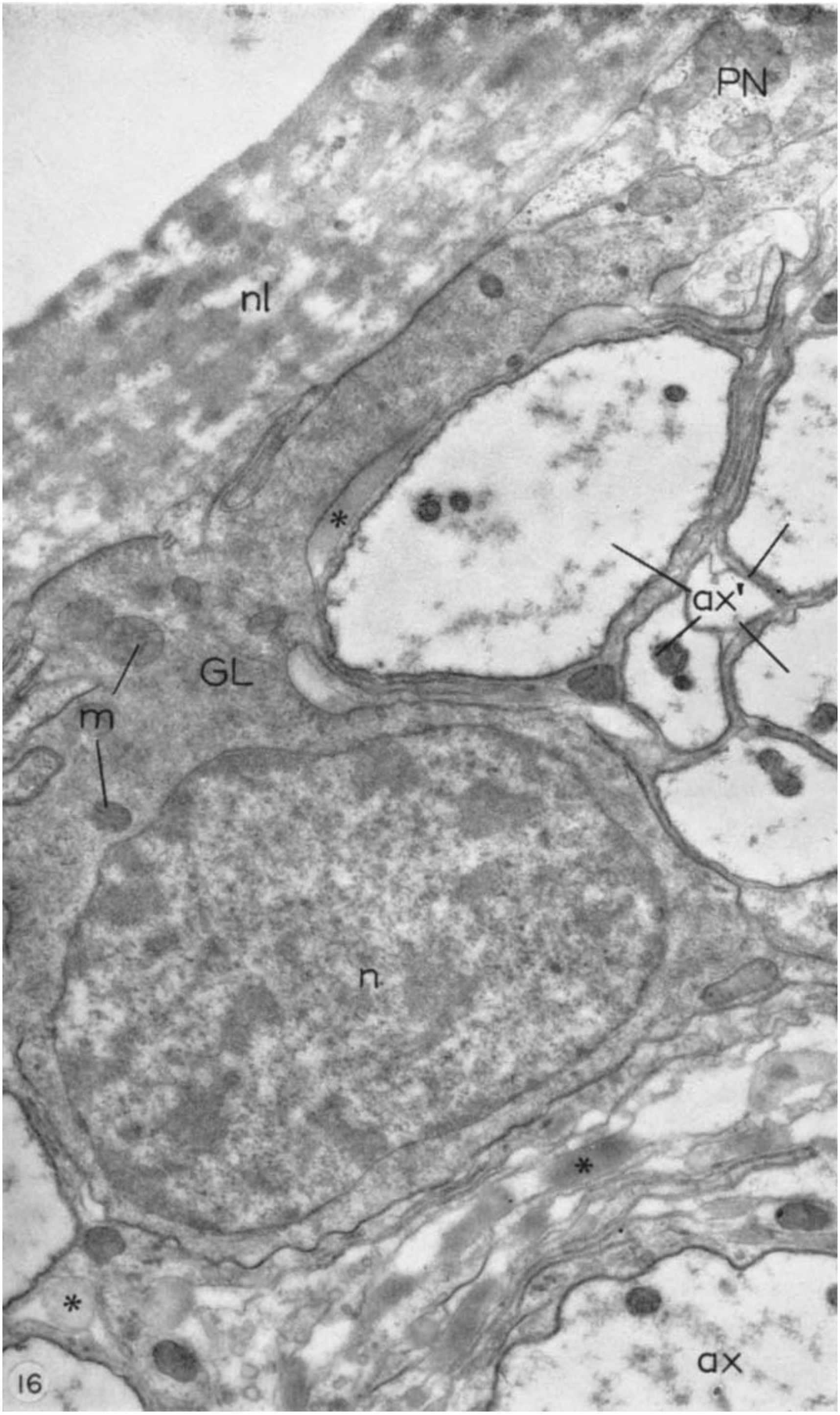
(Wigglesworth, 1959b; Wigglesworth, 1960a), while Hess (1958a) and Treherne and Smith (in preparation) have shown that in the electron microscope the apposed cell surfaces are produced into small-scale interdigitations; a feature illustrated in Fig. 19. Wigglesworth (1960a) has demonstrated the passage of glycogen and lipid to the neurone perikaryon of *Periplaneta*, *via* the invaginated gliocyte processes, and it thus appears that, for these substances at least, a cellular mobilization

pathway is present, linking the perineurium with the cell body of the neurone.

It is convenient at this point to consider the relationship between individual glial cells and their processes, in the peripheral region of the ganglion, and to defer for the moment consideration of the contribution made by glial processes to the organization of the neuropile. The presence of extensive extracellular spaces between the glial cells (the "glial lacunar system" of Wigglesworth) has been confirmed in an electron microscopic study (Treherne and Smith, in preparation). It is clear that the glial region underlying the perineurium in *Periplaneta* includes a complex three-dimensional system of channels lined, in places, with sheets of amorphous dense material of varying thickness (Fig. 13), resembling the extracellular material sometimes accompanying the tracheoles along their incursions into the deeper regions of the ganglion (Fig. 18). In the nerve trunks within the ganglion, in the cercal nerves and in the interganglionic connectives, an apparently similar substance is present between the membranes of the mesaxon folds (Figs. 6, 13, 14, 16, 17). The possible nature of these extracellular components will be considered later (cf. p. 459).

Hess (1958a) and Treherne and Smith (in preparation) observed that a cytoplasmic sheath, albeit narrow, always accompanies the cuticular tube of the tracheoles within the ganglion; the intima of the tracheole is limited on the outside by the superficial plasma membrane of the tracheolar cell prolongation, while against the inner surface lies the membrane across which the tracheolar lining is secreted (Fig. 18). The surface of the tracheolar cytoplasm is overlaid by a basement membrane sheath which, especially in the more extensive glial lacunae, may be confluent with the extracellular material overlying the glial cells. Although the tracheal system is most extensive within the ganglion between the inner and outer glial cell layers, smaller branches (with a lumen diameter of 0·2–0·5 μ) penetrate the deeper layers and are found throughout the neuropile.

FIG. 16. A peripheral field of a transversely sectioned cercal nerve of *Periplaneta*. Beneath the neural lamella (nl) is situated a glial cell (GL): the cytoplasm surrounding the nucleus (n) is dense, and packed with fine fibrils and granules, and contains small numbers of mitochondria (m). This section is close to the point of entry of the nerve into the last abdominal ganglion, and at upper right (PN) occurs a cytoplasmic profile containing numerous mitochondria, probably representing a perineurium extension from the ganglion. The axon at lower right (ax) is ensheathed by a well defined concentric glial covering (cf. Fig. 14) while the group of smaller axon profiles at ax′ are surrounded by a less regular glial system. Note the extracellular material (*) occurring between the membranes of the glial processes. × 18,000.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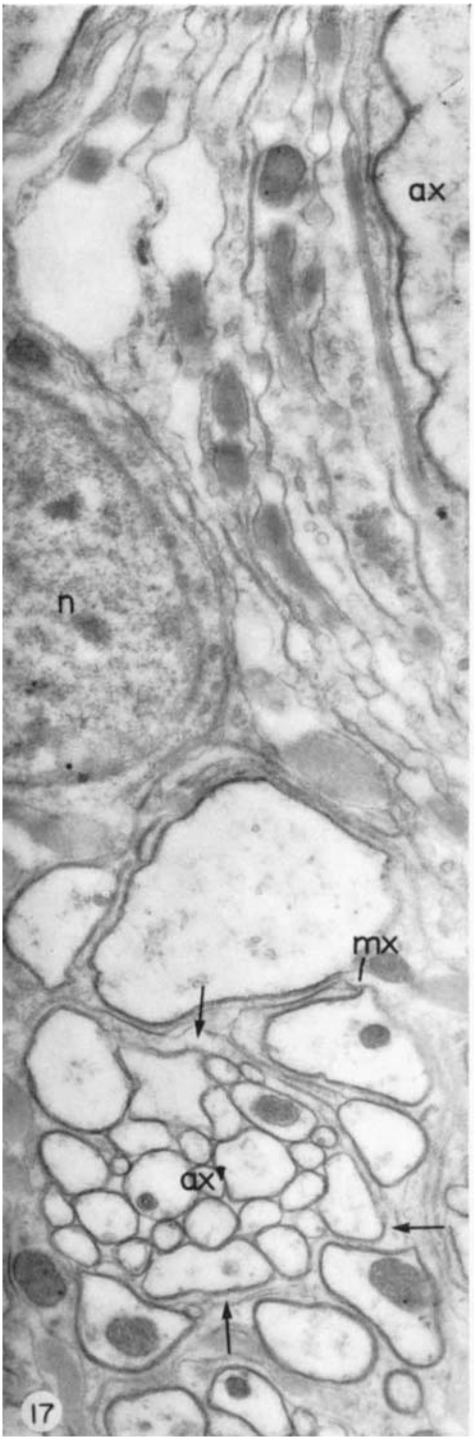
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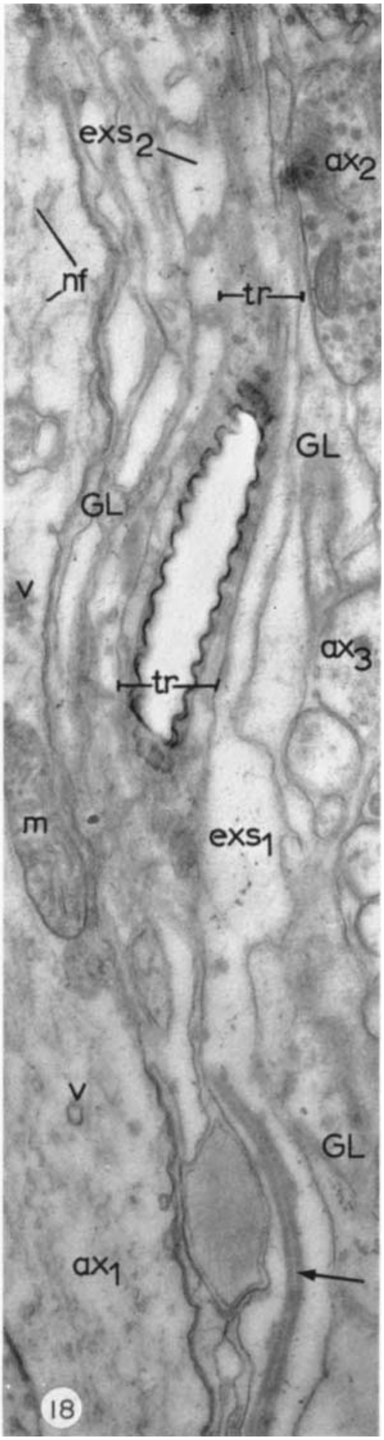
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18

IV. The Neurone

Although the literature on the distribution of neurone cell bodies and axon tracts in insect ganglia is extensive, few accounts are available concerning the detailed cellular organization and interrelations of these components of the nervous system: Wigglesworth (1960b) and Ashhurst (1961a) have investigated the organization of the nerve cell body (perikaryon) of *Periplaneta* with the light microscope, and electron microscopic studies have been made by Trujillo-Cenóz (1959) in *Pholus* (Lepidoptera), by Gray (1959) in *Locusta*, by Trujillo-Cenóz (1962) in *Laplatacris* (Orthoptera) and *Hylesia*, *Automeris* and *Ecpantheria* (Lepidoptera) and by Hess (1958a) and Treherne and Smith (in preparation) in *Periplaneta*, while detailed accounts of the structure of vertebrate neurones have been provided by Palay and Palade (1955), Rosenbluth (1962) and others.

The necessity for careful evaluation of results obtained through histological or cytological work by application of different techniques of fixation and subsequent treatment is obvious, but nowhere more so than in studies on the organization of the central nervous system. For example, the methods of metallic impregnation that have been widely used to visualize the complex topography of the axon tracts within insect ganglia nevertheless, as Wigglesworth (1960b) points out, are quite inadequate for an analysis of the finer cytological details, while, with particular reference to the vertebrate nervous system, "even the magnificently useful method of Golgi derives its usefulness as much from what it does not demonstrate as from what it does"

FIG. 17. A field similar to those illustrated in Figs. 14 and 16, but showing the different spatial relations between nerve and sheath in the case of large and small axons. The axon profile at upper right (ax) is invested with a thick sheath with concentric mesaxon turns, while the cluster of small profiles at ax′ are invested with a common glial sheath (arrows), and mx denotes a mesaxon termination at the surface of a small axon. A gliocyte nucleus is seen at *n*. In the regions of the ganglion outside the neuropile (as in this figure) there is no synaptic association between axon branches, and the axoplasm contains a few small vesicles, neurofilaments, and sparsely distributed mitochondria. ×20,000.

FIG. 18. Illustrating the relationship between tracheal, glial and axonal components in the neuropile of *Periplaneta* ganglion. An obliquely sectioned tracheole tr with its associated cytoplasmic sheath is situated in a narrow extracellular channel exs_1 exs_2, limited on either side by glial cell processes (GL). The latter invest the axon profiles ax_1, ax_2, ax_3 and other small branches. Lamellae of a dense amorphous material lie in the extracellular space in the region indicated with an arrow. Note the neurofilaments (nf), mitochondria (m) and sparsely distributed vesicles (v) in the axoplasm of ax_1, and the concentration of vesicles in ax_2. The latter situation is believed to obtain as the presynaptic axon approaches termination. ×22,000.

(Palay *et al.*, 1962). Furthermore, methods for the demonstration of specific cytochemical components may be unsuited for detailed structural studies. The direct visualization of "bound" osmium in tissue sections by post-fixation treatment of the material with ethyl gallate (Wigglesworth, 1957, 1959a, b) has provided an extremely delicate method whereby the detailed cytoarchitecture of the insect nervous system may be examined in the light microscope. The results of this method give a comprehensive view of the ganglion and other regions, to which electron microscopic studies of similarly fixed material add structural details of cellular organelles and of the minutiae of the spatial relationships between the components of the nervous system.

The object of this preamble is to introduce a necessary word of caution before this account of the insect central nervous system is continued, for the possible inadequacy of present structural concepts must always be borne in mind.

The cell bodies or perikarya or unipolar motor and internunciary neurones lie near the periphery of the insect ganglion, and from them the axon processes lead into the central neuropile. The latter is believed to be the site of much if not all of the synaptic transmission and integration within the insect central nervous system, since the establishment of axo-somatic and axo-dendritic synapses at the surface of the perikaryon, such as occur in the vertebrate nervous system, are here precluded by the glial insulation around this region of the neurone.

Cytological and histochemical studies on the neurone have been made by Wigglesworth (1959b) in *Rhodnius* and by Wigglesworth (1960a) and Ashhurst (1961a) in *Periplaneta*. Certain ultra-structural features of the perikaryon were described by Hess (1958a) and Trujillo-Cenóz (1959) respectively in *Periplaneta* and *Pholus* central ganglia, and by Trujillo-Cenóz (1962) in *Hylesia*, *Ecapantheria* and other insects, and a more extensive investigation of the first of these has been made by Treherne and Smith (in preparation). The organization of sensory neurones associated with the ear of *Locusta* has been described by Gray (1960).

Hess noted that the basiphilic properties of the perikaryon of *Periplaneta* are apparently attributable to the presence of large numbers of RNP granules (ribosomes) which he believed to be free in the cytoplasm, rather than associated with arrays cisternae of the endoplasmic reticulum as in the "Nissl bodies" of the vertebrate neurone (Palay and Palade, 1955; Rosenbluth, 1962; etc.). Gray and Trujillo-Cenóz, however, described both free and membrane-attached ribosome populations. Trujillo-Cenóz (1962) concluded that whereas the ribosome-bearing cisternae of the endoplasmic reticulum often occur in complex whorls

within the perikaryon of scorpions (*Bothriurus*), these are randomly distributed in the case of the insects examined. He also described continuity between the endoplasmic reticulum and the outer membrane of the nuclear envelope: a feature that appears to be of general occurrence in many cell types. Each of these authors recognized the presence of small groups of agranular cisternae forming associations similar in shape and appearance to the "Golgi bodies" or "dictyosomes" described by many authors in light and electron micrographs of invertebrate neurones and vertebrate and invertebrate germ cells, and they noted that mitochondria are distributed throughout the perikaryon cytoplasm.

In the preparations described by Hess, the cell bodies fell into two categories, the "light" and "dark" cells: in the former the RNP particles were found to be aggregated into clusters in a cytoplasmic matrix containing many large "empty" regions, while in the latter these ribosomes were distributed more evenly throughout the more compact cytoplasm. This type of variation was also observed by Wigglesworth (1960b) in osmium-ethyl gallate preparations of the ganglia of *Periplaneta*, but not by Ashhurst (1961a) in a histochemical study of the same material.

In a reinvestigation of the cytological organization of the last abdominal ganglion of *Periplaneta*, Treherne and Smith found that preservation of neurones and other cellular components is poor, if fixation and embedding is carried out on intact ganglia: possibly the fixed perineurium slows down the penetration of osmium to the deeper layers. A striking improvement is achieved if the freshly exposed ganglion is irrigated with a drop of fixative, quickly exised, and very carefully bisected before being replaced in the fixative. Profiles of perikaryon cytoplasm thus treated are illustrated in Figs. 19 and 20. The bulk of the cytoplasm contains subcircular or angular profiles of membrane-limited cisternal elements. These may be free from or associated with particles presumed to be ribonucleoprotein, corresponding respectively to the "smooth" and "rough surfaced" endoplasmic reticulum components (Fig. 19). As was noted by Hess and Gray, unattached RNP particles are also profusely distributed in the cytoplasm. Circular or elongated profiles of mitochondria occur throughout the perikaryon, and "multivesicular bodies" are sometimes seen (Fig. 19). The latter were described by Trujillo-Cenóz (1962) in insect perikanya and were found to be more abundant in the scorpion.

In addition to the structures mentioned above, the perikaryon cytoplasm of the *Periplaneta* neurone contains large numbers of highly

ordered membrane associations corresponding to the structures that have commonly been described as "dictyosomes" or "Golgi bodies". The morphology of these, as seen in the material described by Treherne and Smith (in preparation), will first be described, and following this the controversial question of their nature and affinities will be discussed. These associations appear as arrays of straight, or more often, crescentic or U-shaped series of agranular membrane-limited cisternae; each cisterna encloses an electron-transparent cavity, *c.* 100–200 Å in width (Fig. 20). In these complexes, the cisternal profiles often have a beaded appearance. On either side of the cisternae, but especially enclosed within the arms of the crescentic associations, are seen large numbers of smooth-membraned circular profiles, consistently smaller than those distributed elsewhere throughout the perikaryon, and these frequently containing dense inclusions. Similar dense inclusions are sometimes seen within the cisternae, and it is possible that the discrete vesicles represent membrane-limited units enclosing materials initially sequestered within the cisternal cavities, that are split off the parent cisternae in the manner described by Scharrer (1961) in neurosecretory neurones of *Lumbricus*. Similar vesicles were noted in *Ecpantheria* by Trujillo-Cenóz (1962) who suggested that they may be elaborated within the "Golgi region", and possibly also in association with the multivesicular bodies. In addition to these small vesicles, the dictyosome region in *Periplaneta* often contains large osmiophilic bodies (Fig. 19). Occasionally, tangential profiles of these cisternae are met with (Fig. 20), which indicate that each is interrupted by circular perforations, *c.* 40–70 mμ in diameter; a type of organization resembling that of the "annulate lamellae" described by Rebhun (1956) in the cytoplasm of invertebrate

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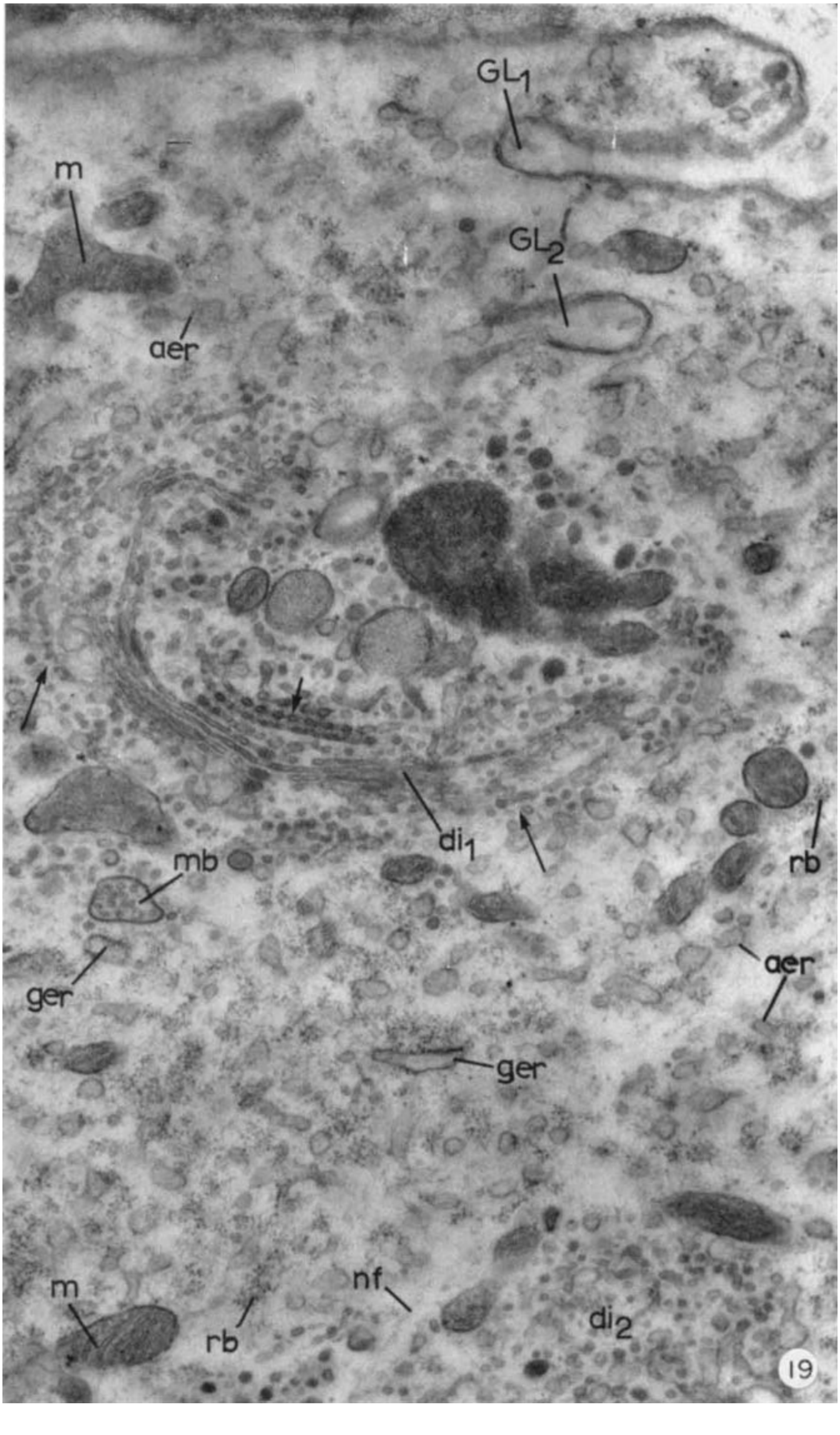
FIG. 19. Illustrating the cytoplasmic organization of the perikaryon (cell body) of a neurone in the ganglion of *Periplaneta*. At upper right a finger-like glial invagination (GL_1) indents the neurone surface, while the profile indicated by GL_2 probably represents a transverse section of a similar process. The cytoplasmic matrix of the neurone contains large numbers of profiles of elements of the agranular endoplasmic reticulum (aer), a few ribosome-bearing cisternae of the rough-surfaced or granular reticulum (ger) and clusters of unattached ribosomes (rb). Mitochondria (m) multivesicular bodies (mb) and small numbers of neurofilaments (nf) are present.

A dictyosome profile (di_1) occupies the centre of the field: this comprises crescentic stacks of agranular cisternae often exhibiting a "beaded" appearance (short arrow) (cf. Fig. 20). At the lateral margins of this complex structure the cisternae appear to be fragmenting into a swarm of small vesicles (long arrows), which may have a dense content. Similar vesicles and larger bodies showing varying degrees of osmiophilia lie in the centre of the dictyosome region. The edge of a second dictyosome (di_2) appears at lower right. $\times 25{,}000$.


GL1
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oocytes. Transverse or oblique sections of cisternae thus organized apparently afford the "beaded" profiles (Fig. 19) mentioned above.

Mitochondria are often, though not invariably, aggregated in the dictyosome region. The fine cytoplasmic "neurofibrillae" described by Palay and Palade (1955) in the vertebrate perikaryon also occur in *Periplaneta*, where they are of similar diameter (50–100 Å) but apparently sparsely distributed (Fig. 19).

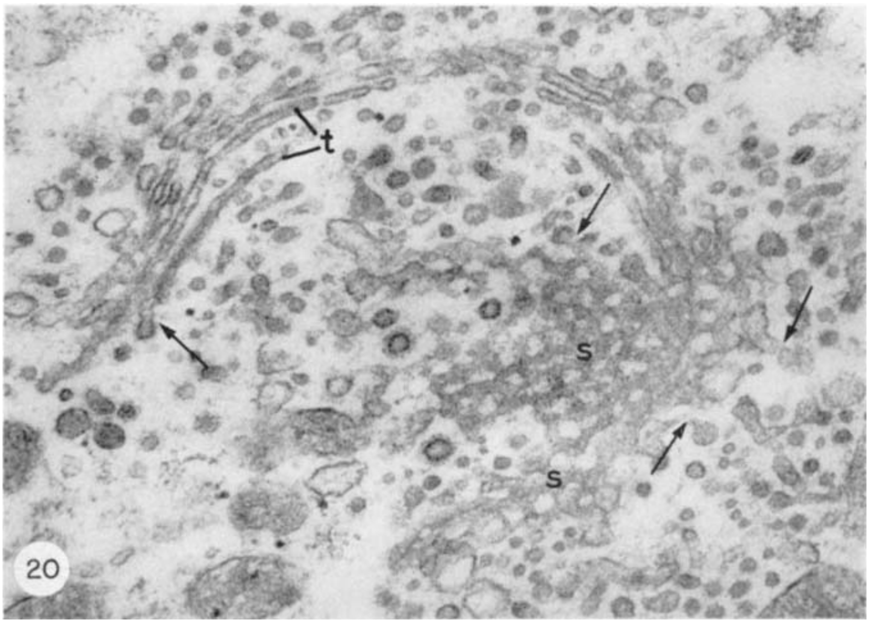


FIG. 20. A dictyosome in the perikaryon of *Periplaneta* similar to that illustrated in Fig. 19, including cisternae in transverse section (t) and a portion of a cisterna seen in surface aspect (s). The latter is seen to be perforated by subcircular holes, presumably giving rise to the "beaded" appearance of profiles perpendicular to this plane of section (cf. Fig. 19). In some places (arrows) small vesicles appear to be fragmenting from the main cisternal complex. ×45,000.

The previously mentioned "light" and "dark" perikarya of Hess were also noted by Treherne and Smith. Hess contended that even if the extensive cytoplasmic discontinuities characterizing the "light" cells are introduced during preparation, the occurrence of both types in the same region of the ganglion seems to indicate a genuine distinction between them. Treherne and Smith found, however, that graduations occur between these two extremes; that variation is restricted to the extent

of cytoplasmic discontinuity only, and that this feature is absent in neurones judged to be well preserved. It is possible that the perikaryon is unusually sensitive in its response to the procedures of fixation, dehydration, or embedding, for examination in the electron microscope.

The cytoplasmic organization of the neurone cell body in the insect ganglion changes rather abruptly in the region of transition between the perikaryon and the axon—the "axon cone". In particular the axoplasm lacks the extensive system of granular cisternae and unattached ribosomes characteristic of the cell body, and the restriction of these components to the perikaryon has been shown by Trujillo-Cenóz (1959, 1962) and Gray, (1959). One of the most important questions, albeit at present obscure, concerning the physiology of the neurone is that of the metabolic relationship between the axon process and the neurone cell body. By analogy with other cells it seems probable that many of the synthetic functions of the neurone cell are associated with the endoplasmic reticulum elements within the perikaryon; as Wigglesworth (1960a) has shown, at least one of the functions of the complex cell body–gliocyte relationship is the transfer of nutrients to this region of the neurone, and there is believed to be a flow of products from the perikaryon along the axon processes (Koenig, 1958). The question of the existence of a structural pathway along the insect neurone, linking the perikaryon with the axon, cannot at the moment be decided on the basis of light and electron microscopic studies. Many light microscopic studies have revealed a fibrillar system in the axoplasm and perikaryon of the insect neurone (Cajal and Sanchez, 1915; Monti, 1913; Beams and King, 1932; Pipa and Cook 1958, etc.); these appear to correspond to the "neurofibrillae" of vertebrate nerve cells. Although particularly evident in silver-impregnated material, similar structures have also been reported in phase-contrast preparations of *Periplaneta* ganglia: the axoplasm of large and small axons was found to be packed with unstained canals or fibrils, *c.* 0·5 μ in diameter, fanning out within the perikaryon, within which each canal appeared to be associated with a dictyosome, and Wigglesworth concluded that the latter are concerned in the secretion of "fibrils of axoplasm". This conclusion was supported by his observation that post-section regeneration of the nerve is accompanied by morphological changes within the perikaryon that possibly indicate increased activity of the dictyosomes.

Wigglesworth suggested that the neurofibrils he observed may correspond to bundles of the fine "neurofilaments" first described in electron micrographs by Palay and Palade (1955), and he related the

extensions of the unstained components into the cell body to the aggregations of neurofilaments described by Palay and Palade in the corresponding region of mammalian neurones. The appearance of the large axoplasmic channels in Wigglesworth's preparations is very striking, and these may well be the structures outlined by deposits of silver in impregnated material; their organization does not, however, appear to be preserved in conventional electron microscopic preparations. Treherne and Smith (in preparation) found that the axoplasm in

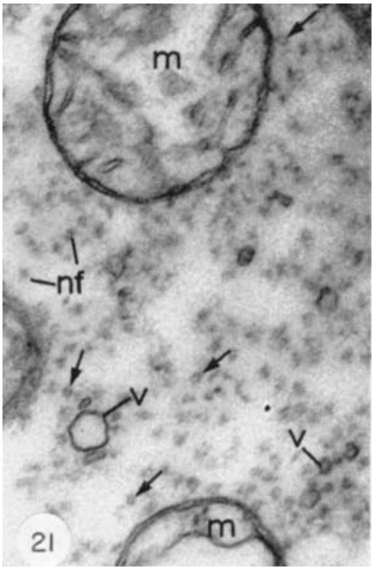


FIG. 21. Illustrating the organization of the axoplasm in a transversely sectioned non-synaptic region of an axon in the neuropile. Mitochondria (m) are present, and neurofilaments, 150–250 Å in diameter are abundant, and the latter sometimes appear to be tubular (arrows). A few larger membrane-limited vesicle profiles (v) also occur in the axoplasm. ×40,000.

Periplaneta ganglia contains narrow oriented filaments or tubules, *c*. 200 Å in diameter (Fig. 21), similar to those found in small numbers within the perikaryon, but these are not gathered into axoplasmic tracts. These "neurofilaments" appear to be less precisely outlined than are the membranes defining other cell components, and they are distinct from larger membrane-limited tubules and vesicles, probably referable to the endoplasmic reticulum, occurring sparsely throughout the axoplasm (Fig. 21) in *Periplaneta*, and in other insects (Trujillo-Cenóz, 1959, 1962). Wigglesworth points out that while the axons

in the centre of the neuropile (the main synaptic region) are stained darkly with osmium-ethyl gallate, presumably by virtue of the concentration of membrane-limited vesicles in these regions (Fig. 22), elsewhere the canals in the axons are unstained, suggesting that they neither contain appreciable quantities of the lipid occurring in cytoplasmic membranes elsewhere, nor are they limited by such membranes. The nature of the insect "neurofibrils" and "neurofilaments" is thus at present somewhat problematical: on the basis of the appearance of the latter in the electron microscope, it cannot be said that they have been shown to form an organized system, linking the neurone cell body with axon extremities. This may, however, be due to inadequate preservation of available material, for there is elsewhere, in vertebrate neurones (Webster, 1962; Palay *et al.*, 1962), convincing evidence for association between neurofilaments and vesicular axoplasmic bodies believed to represent elements of the endoplasmic reticulum: to form a system along which the materials elaborated in the perikaryon may be channelled down the axon processes.

A. THE AFFINITIES OF THE "GOLGI BODIES" AND "DICTYOSOMES" IN THE INSECT NEURONE

It is beyond the scope of this account to review the controversy that has centred around the identity of the "Golgi apparatus" since the first description of an "endocellular reticular apparatus" revealed by silver impregnation in Purkinje cells of the cerebrellar cortex of an owl, described by Golgi (1898). Subsequent light microscopic work pointing, on the one hand, to the ubiquity of systems believed to be homologous with that described in the classical account, and, on the other, to the possibility of the "Golgi apparatus" being an artifact, has been reviewed by Nath (1926) and Hirsch (1939), and recent work, including electron microscopic evidence, has been reviewed by Dalton (1961) and Hirsch (1962). The history of the controversy epitomizes the difficulties inherent in relating results of histochemical or cytochemical techniques to the structural organization of the living cell. The advent of electron microscopic techniques, permitting the examination of thin sections of biological material, afforded not only a picture of the cell at high magnification and resolution, but also introduced the possibility of structural identification of cellular components previously characterized on the basis of their response to staining or other techniques. However, the structural and histochemical classifications did not always correspond, and the electron microscopist inherited the "Golgi

controversy" from his predecessors, and it is evident that a solution to the problem can only be expected from unified studies employing these respective techniques. The aim of the present account is to relate the structure of the insect neurone cell body to that of other animals, and in particular to compare the results of light and electron microscopic studies, as a contribution to the solution of the problem of neurone organization in general.

The classical internal "Golgi apparatus" denoted an intracellular network of fine filaments and larger bodies, situated within the neurone cytoplasm, and demonstrable by silver-impregnation methods. Malhotra (1959, 1960) and David *et al.* (1960) demonstrated, in the interference microscope, a network within the living vertebrate neurone that agreed in its distribution with the silver-stained image of the classical "Golgi apparatus", and also with the basiphilic "Nissl substance". In electron micrographs the latter has been found to correspond to the RNP-particle studded (granular or "rough surfaced") cisternae of the endoplasmic reticulum (Palay and Palade, 1955; Malhotra and Meek, 1960), and it appears that the "Golgi apparatus" as originally described in the vertebrate perikaryon represents the site of silver deposition on the membrane of the endoplasmic reticulum, deployed in an interconnected system throughout the cell. Malhotra (1961) and David and Brown (1961) found that after suitable pretreatment designed to "unmask" lipid material, a sudanophilic system corresponding to the silver-impregnated "Golgi apparatus" and to the basiphilic "Nissl substance" is visualized, and this presumably represents the membranous superstructure of the endoplasmic reticulum of the perikaryon. In invertebrate neurones and vertebrate and invertebrate germ cells, the classical silver-stained "Golgi apparatus" appears not as a reticulum but as a series of discrete bodies, the "dictyosomes", distributed throughout the cytoplasm. The immediate problem to be settled is the relationship between these structures and the "Golgi apparatus" of vertebrate neurones as identified above. The "dictyosomes" of invertebrate neurones have been described in light microscopic preparations by many authors, and specifically in insect neurones by Shafiq (1953) and Shafiq and Casselman (1954, *Locusta*), Beams *et al.* (1953, *Melanoplus*), Gresson *et al.* (1956, *Locusta*), Malhotra (1956), Ashhurst (1961a, *Periplaneta*), Wigglesworth (1960b, *Periplaneta*) and others. The organization of the perikaryon of *Periplaneta* as seen in electron micrographs (Treherne and Smith, in preparation) has already been described; in this material it was found that the cytoplasm contained large numbers of free ribonucleoprotein particles, and relatively few "rough surfaced"

cisternae of the endoplasmic reticulum; a striking deviation from the situation met with in vertebrates, and an observation that is in accord with the uniform basiphilia and absence of "Nissl bodies" noted by Malhotra (1960) and Ashhurst (1961a), respectively, in neurones of crustacea and *Periplaneta*, and by Moussa and Banwahy (1959) in *Schistocerca*.

The crescentic profiles of the "dictyosomes" described by Wigglesworth (1960b) and others are easily related to the complex groupings of agranular cisternae described by Treherne and Smith in electron micrographs of *Periplaneta* perikarya, and by Trujillo-Cenóz (1962) and others in various insects and other arthropods, and the parallel arrays of cisternae described in the latter accounts have often been described as "Golgi bodies" or "Golgi elements". It is clear that according to the classical account of the "Golgi apparatus" this is a misnomer, since the original term evidently relates to the rough-surfaced endoplasmic reticulum of the neurone. Chou and Meek (1958), Ashhurst (1961a) and others, have suggested that the crescentic form of the "dictyosomes" is an artefact, produced by the deposition of osmium (or silver) on spherical bodies stained with neutral red in the living neurone, corresponding to the "vacuome" of Parat and Painlevé (1924a, b, c). The former authors have presented evidence that phospholipid deposits in the neurones of *Helix aspersa* may be transformed by the mode of fixation into the arrays of agranular cisternae representing the "dictyosomes". However, Dalton (1960; cited by Dalton, 1961) was not able to confirm these results in the allied species *Helix pomatia*, and in the perikaryon of the neurone of *Periplaneta* described by Treherne and Smith (in preparation) both complex cisternal associations corresponding precisely to the light microscopic images of "dictyosomes" described by Wigglesworth (1960b) and others, and osmiophilic membrane-limited "droplets" were observed. Moreover, Trujillo-Cenóz (1962) has shown that in the perikaryon of the scorpion *Bothriurus*, the agranular cisternae may be morphologically continuous with elements of the ribosome-bearing portion of the endoplasmic reticulum.

There is thus little reason, on the available evidence, for dismissing the highly organized dictyosomes of the invertebrate neurone as artefacts. Indeed, there is good evidence that the association of agranular membranes constituting the dictyosome plays an important part in the secretory activities of the neurones: Dalton (1961) and Scharrer (1961) have described sequestration of neurosecretory material within the cisternae of the dictyosome, and the splitting off of membrane-limited droplets from the main complex. In addition, it may well be

that other products, cytologically less clearly characterized than the dense neurosecretory droplets, are elaborated in the cisternae of the dictyosome for transport along the axon processes.

Together with the granular cisternae of the endoplasmic reticulum and the unattached ribosomes, the dictyosomes of the neurone of insects and other invertebrates may be the counterpart, in terms of the synthetic activities of the cell, of the morphologically distinct "Nissl- or Golgi system" of the perikaryon of the vertebrate neurone.

V. The Neuropile

The medullary or central portion of the insect ganglion forms an anatomically well-defined region from which the neurone cell bodies are excluded, and consists of a complex association of sensory, motor and internunciary axon processes together with gliocyte extensions, within which the synaptic pathways of the central nervous system are established.

Much work has been carried out on the gross anatomy of the neuropile; methods of silver impregnation have revealed the distribution of the larger axons and tracts, and the osmium-ethyl gallate method of Wigglesworth (1957, 1959b) demonstrates many features of the structure and relationship of the components of the neuropile down to the limit of resolution of the light microscope. It is upon this last account, and the electron microscopic studies of Hess (1958a), Trujillo-Cenóz (1959, 1962) and Treherne and Smith (in preparation), that our present limited understanding of the cytological architecture of the insect neuropile principally rests.

The definitive feature of a synaptic junction within the central nervous system is that a functional link is present between specific regions of two nerve cells, across which a signal supplied by one, the presynaptic element, elicits a response in the other, the postsynaptic element; the two elements being separated by a narrow extracellular space, the "synaptic cleft" or "synaptic gap". Elucidation of the morphological relationship between the synaptic components was formerly limited by the resolution of the light microscope, and by the preparatory techniques employed. The development of electron microscopic methods permitted the analysis of the structural integration of the nervous system at a new level and, of particular importance in the present context, provided much information concerning the morphology of the synapse. Palay (1958) and de Robertis (1958, 1959) have given valuable reviews of the electron microscopic evidence on the subject, and it is clear that striking

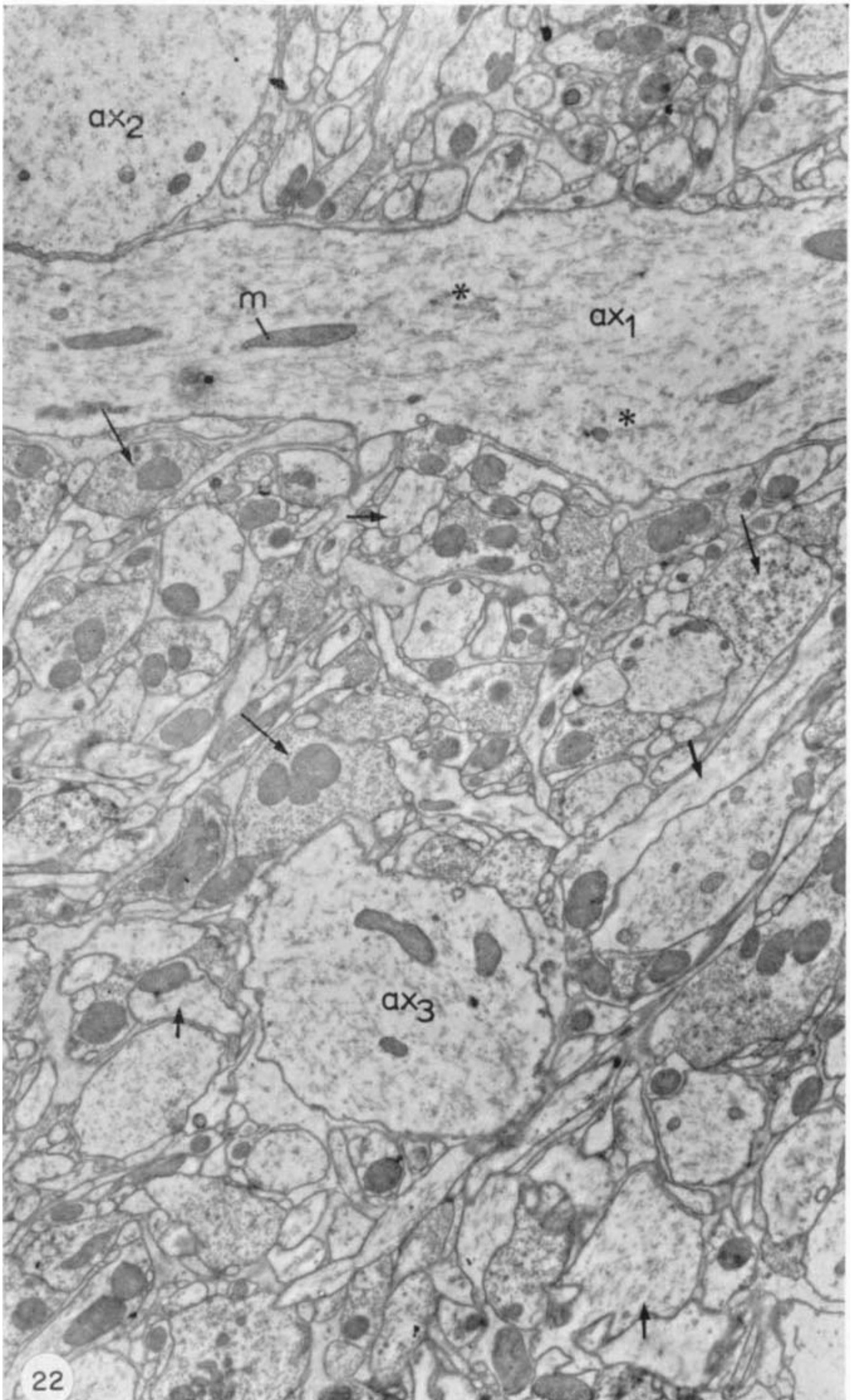
similarities exist between synapses throughout the nervous system, whether in vertebrates or in invertebrates. In all instances the pre- and postsynaptic (or "subsynaptic") membrane surfaces are separated by a gap, generally about 100–200 Å in width, from which neuroglial or other cell processes are excluded, and the cytoplasm of the presynaptic region usually contains large numbers of membrane-limited vesicles, *c*. 200–600 Å in diameter, first described by de Robertis and Bennett (1955), Palade (1954) and Palay (1954) and subsequently by many authors. While occasionally, as in the giant fibre-to-motor synapse in the crayfish central nervous system (Furchpan and Potter, 1959), the presynaptic action potential appears to be transferred directly by ion flow across the synaptic gap to the postsynaptic side of the junction, in the majority of instances the initiation of a propagated postsynaptic potential is believed to be mediated by the release from the presynaptic membrane of a chemical transmitter (acetylcholine or a functional analogue) that diffuses across the extracellular space separating the two components of the synapse. The transmitter exerts its effect by altering the ionic permeability of the postsynaptic membrane, and is afterwards hydrolysed enzymatically. The results of electron microscopic studies both of the central nervous system and of the nerve-muscle junction, have been integrated with certain physiological features in a very attractive hypothesis (de Robertis, 1959; Katz, 1962) centering around the mode of discharge of transmitter substance, believed to be sequestered in the vesicles concentrated in the presynaptic axoplasm, into the synaptic gap. This hypothesis will be discussed more fully in a later section dealing with the insect myoneural junction, but it may be pointed out here that available evidence suggests that in insects, as in vertebrates, the discontinuities between the excitable components in the central nervous system and at the motor ending on a muscle fibre may be functionally bridged in a similar manner.

The generalized insect nervous system (Wigglesworth, 1953) is compounded of bipolar sensory neurones, the cell bodies of which lie outside the central ganglia close to the receptor site, and motor and internunciary (association) neurones with cell bodies situated at the edge of the neuropile within the ganglia. The last two neurone types are generally monopolar; the motor axons leading from the cell body initially pass into the neuropile, where they may form synaptic associations *via* side branches (collaterals) with axons of internunciary and sensory neurones, before they pass out of the neuropile and the ganglion to the effector system. Cajal and Sánchez (1915) concluded, on the basis

of comparative studies on nervous systems, that whereas axo-somatic and axo-dendritic synapses are profusely distributed over the surface of the cell body and its processes in many vertebrate neurones, the insect neurone cell body is devoid of such synaptic contacts, which take place exclusively within the neuropile, and it has been assumed that much or all the synaptic transmission within the latter takes place across the complex interlaced branches ("terminal arborizations") into which the axons and their collaterals divide.

Detailed study of the insect neuropile by light microscopic methods is rendered extremely difficult by the small size of the components within it. The electron microscopist presents himself with images of the neuropile at high resolution and of such complexity that, at the moment, they may only be analysed in general terms. The only electron microscopic descriptions of this region of the insect ganglion are those of Trujillo-Cenóz (1959) in the Lepidopteran *Pholus* and other species (1962), and of Hess (1958a) and Treherne and Smith (in preparation) in *Periplaneta*. Certain features of the organization of the neuropile in the latter species are illustrated in Figs. 18, 21–26. Throughout this portion of the ganglion, transverse, longitudinal and oblique profiles of axons and their branches abound (Fig. 22): throughout the greater part of the neuropile these profiles are rather closely packed, and neuroglial cytoplasm is relatively restricted in extent. Outside the peripheral zone of the neuropile, however, axon profiles with well-developed glial sheaths occur (Figs. 14, 17), and these appear to correspond to the pseudomyelinated axons, described by Wigglesworth (1959b), in osmium-ethyl gallate preparations, as having a darkly-staining sheath, exhibiting a concentric structure. In electron micrographs, this sheath is found to conform to the "tunicated" arrangement observed in peripheral nerves of insects (Edwards *et al.*, 1958a; Wigglesworth, 1959a; Hess, 1958b, etc.) and of scorpions (Trujillo-Cenóz, 1962): one or more mesaxons are invaginated from the surface of the glial cell and are arranged around the axon in a more or less concentric fashion.

FIG. 22. A low-power survey electron micrograph of a field in the neuropile of the last abdominal ganglion of *Periplaneta*. Most of the axon branches present are seen in transverse section: that at ax_1 is sectioned longitudinally and contains a few vesicles (*), small elongated mitochondria (m) and large numbers of neuro filaments. The axoplasm of the large profiles at ax_2 and ax_3 is similar. Elsewhere occur many smaller axon branches, some containing neurofilaments and small mitochondria (short arrows) and others containing mitochondria and concentrations of vesicles (long arrows). The detailed organization of these nerve components, and their relationship with the glial processes is illustrated in Figs. 21, 23, 24, 25, 26. ×7,000.



ax2
m
*
ax1
*
ax3
22

Whereas in a myelinated fibre the successive "turns" of the mesaxon form a compact system of lipoprotein membranes, in this instance layers of glial cytoplasm are present throughout the sheath. Dilatations or "lacunae" in the mesaxon system have been described in insect peripheral nerves (Edwards *et al.*, 1958a, b; Hess 1958b; Smith, 1960), and this system is exceptionally regular and well developed in the tunicated axons of the central ganglia of *Periplaneta* (Fig. 14) and also in the axons of the cercal nerves (Fig. 16) and inter-ganglion connectives. In transverse sections, the dilatations are elongated or lenticular, up to 200 μ in width; elsewhere the membranes constituting the mesaxon are separated by a space of *c*. 100–200 Å. Within most of the dilatations occurs a profile of homogeneous material, more electron-dense than the surrounding glial cytoplasm, sometimes continuing for some distance along the cavity between the membranes (Figs. 12, 14, 16). The innermost fold of the mesaxon, however, always follows the contours of the axon plasma membrane, from which it is separated by a rather uniform space *c*. 100 Å wide (Fig. 14). It appears that this narrow extracellular gap around the axon is continuous with an extensive extracellular system enclosed within the external limits of the glial cell and lying between the mesaxon membranes. As Hess (1958b) noted, the smaller axons may be associated in groups with processes of a single gliocyte: in this situation (Fig. 17) the relationship between axon and sheath described above is preserved, but the concentric arrangement of the latter is often absent. The cytoplasm of the glial processes around the axons in this region of the ganglion contain small profiles, predominantly of the agranular endoplasmic reticulum, either scattered or grouped in small subparallel arrays, while the axoplasm contains neurofilaments *c*. 200 Å in diameter, a few profiles of vesicular or tubular elements and sparsely distributed small mitochondria (Fig. 21) exhibiting a very simple crista arrangement.

In the region bordering on the neuropile, described above, the glial cells are loosely distributed, and more or less extensive extracellular spaces occur between them, corresponding to the "glial lacunar system" of Wigglesworth (1960a). The appearance of such a lacuna or sinus in the electron microscope is seen in Fig. 13: the superficial glial plasma membrane bordering on the intercellular cavity has only a faintly indicated continuous basement membrane, but bulky and often irregular deposits of material of similar density to that found in the mesaxon dilatations are scattered throughout the lacuna, either as sheets or bands following the contours of the glial cells for some distance, or else lying free within the space between them. Smaller amounts of similar material

accompany the gliocyte processes and the tracheoles as they course deeply into the neuropile; occasionally lamellar deposits occur (Fig. 18), and it is possible that this material corresponds to the "glial tubuli" believed by Trujillo-Cenóz (1959) to be intracellular structures, although it appears that the system he described in *Pholus* is more organized and extensive than in *Periplaneta*. It is important to note that no trace of collagen-like fibrils have been detected in this extracellular material, and its nature is unknown. It may, however, represent the PAS-positive substance described by Wigglesworth (1960a) as occurring around the axons and neurone cell bodies lining the glial lacunae and elsewhere, for in each location this extracellular material has been recognized between the glial cells. On the other hand, it is possible that this material is related to the acid mucopolysaccharide component described by Ashhurst (1961b) and others as occurring in this region of the ganglion. On the electron microscopic evidence, only its non-collagenous nature seems certain.

It is clear from electron microscopic studies and from Wigglesworth's light microscopic work that extensive glial sheaths are lost when the axons enter the neuropile. Trujillo-Cenóz (1959) overlooked the neuropile glial system and regarded all the axons within it as being devoid of any insulation restricting contact between them, though in a more recent paper (1962) he suggested that although glial processes were not identified with certainty in the neuropile region of several insects and other arthropods examined, these may be admixed with the profiles of the nerve fibres and their branches. Hess noted that the larger axons are often invested with a narrow glial sheath associated with a very short mesaxon, and that narrow sleeves of membrane-limited glial cytoplasm are seen to be interposed between the adjoining axon surfaces. In the regions of the neuropile occupied by smaller axon branches, a similar tenuous system of glial ramifications is present (Fig. 24), but gliocyte cell bodies never occur within the neuropile: these lie adjacent to those adjoining the neurone perikarya in the more peripheral regions of the ganglion.

Throughout the neuropile are found numerous axon profiles, the plasma membranes of which are separated by a space of only *c.* 100–200 Å. Within any small area in the deeper regions of the neuropile, gliocyte arborizations are seen to separate some axon profiles, while by-passing others (Fig. 24). If it is assumed, by analogy with other central nervous systems (de Robertis, 1959), that interpolation of glial cytoplasm in the insect neuropile precludes the establishment of a synaptic relationship between two axon processes, and, furthermore,

that unrestricted apposition *may* indicate a synaptic region, then the glial processes in the neuropile can be visualized as a topographically extremely complex system dissecting the multitude of axon processes into functional pathway, blocking or permitting synaptic transmission respectively by its presence or absence, and thus defining synaptic pathways in the organization of which resides the integrated function of the central nervous system.

While there is every reason to believe that the establishment of a synapse necessitates the close apposition of pre- and postsynaptic membranes, it cannot be assumed *ipso facto* that all such regions of apposition in the neuropile are synapses, for the actual site of synaptic transmission may be very restricted. This appears to be so, for example, in many regions of the vertebrate central nervous system (see de Robertis (1959) for discussion and references). In the latter instance, the morphology of pre- and postsynaptic elements may have a characteristic distribution, as in the case of the axo-somatic and axo-dendritic *boutons terminaux* associated with the perikaryon of the neurone and its dendrites, but in the neuropile, with its formidable tangle of axons, the recognition of synaptic regions is a far more difficult task.

Comparative studies on the cytology of the central nervous system have suggested that certain features are usually met with in the region of synaptic junction between two nerve cells. In particular the presynaptic axoplasm often contains large numbers of vesicles, and a higher concentration of mitochondria than elsewhere, and the former are

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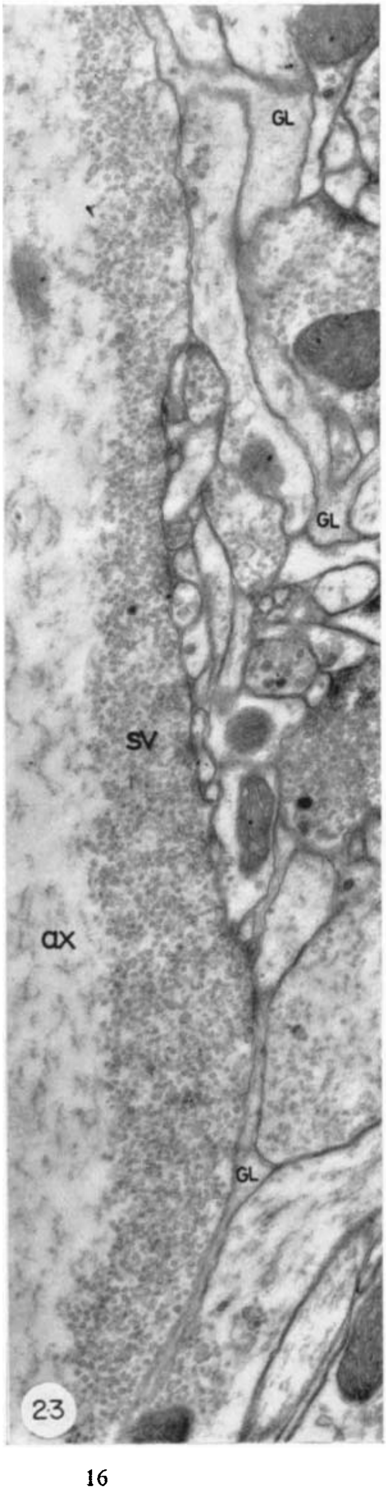
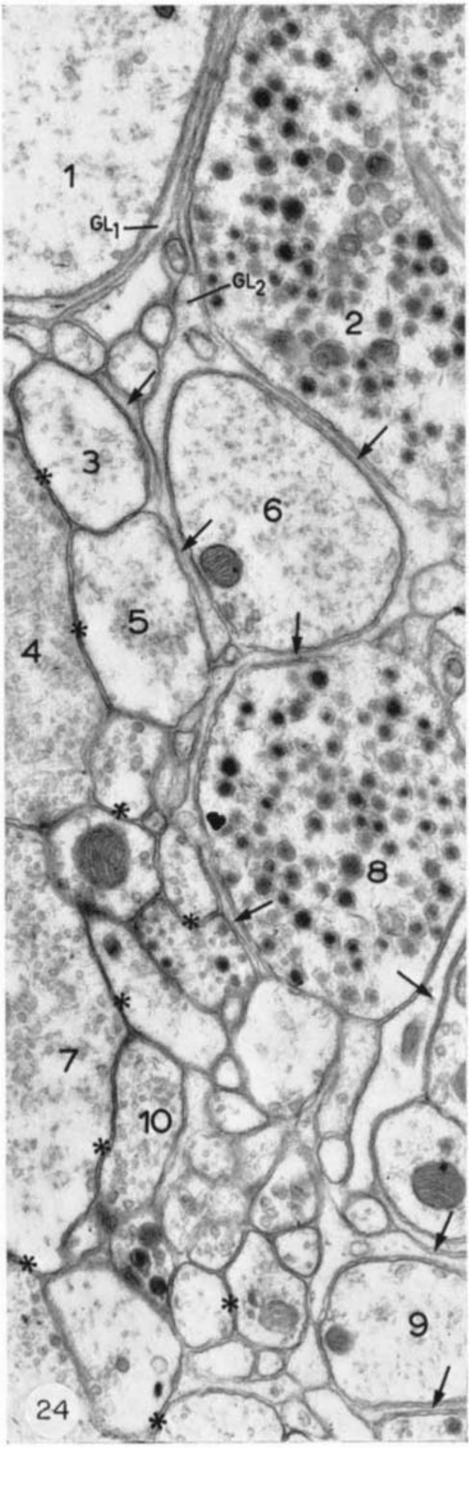
FIG. 23. In this figure, the longitudinally sectioned axon *ax* contains large numbers of synaptic vesicles (sv) concentrated along one side. Closely applied to this are numerous small profiles of axon branches. It is possible that some of the latter represent fine post-synaptic terminals ending on a larger presynaptic component (ax). Elsewhere, axon profiles are present some containing synaptic vesicles and others lacking these structures (cf. Fig. 25), and a few glial cell processes may be identified in this field (GL). ×17,000.

FIG. 24. A micrograph of a region in the neuropile, illustrating the fine arborizations of the glial system. The axons at upper left and right (1, 2) are invested with distinct glial sheaths (GL_1, GL_2), and elsewhere glial cell processes lying between adjoining axon profiles are indicated with arrows, and some regions of axon apposition from which glial material is excluded, by asterisks. There is no reason to suppose that absence of glial insulation alone is diagnostic of a synaptic region; characteristic associations of vesicles and dense regions along the presumed synapsing surfaces believed to represent active sites of synapse occur in the neuropile (Fig. 26), but are not present in this field.

Note the membrane-limited droplets, probably of neuro-secretory material, present in large numbers in the axoplasm of branches 2 and 8. Axons 1, 3, 5, 6, 9, etc. contain neurofilaments and small numbers of vesicles, while the presence of large concentrations of vesicles in axons 4, 7, 10, etc. is believed to indicate that they are pre-synaptic branches approaching termination. ×25,000.


GL
GL
SV
ax
GL
23


1
GL_1
GL_2
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24

sometimes preferentially associated in clusters opposite localized regions of high electron density situated at intervals along the presynaptic side of the apposed synaptic membranes (de Robertis, 1959). Localized aggregations of bodies resembling the "synaptic vesicles" first described by de Robertis and Bennett (1955), Palade (1954), Palay (1954) and subsequently by many investigators, in central nervous synapses and in the terminal axoplasm of the myoneural junction, have been observed in the axons of the insect neuropile by Hess (1958a), Trujillo-Cenóz (1959, 1962) and Treherne and Smith (in preparation), and there is now good evidence (which will be outlined later) that the organization of motor endings on muscle fibres in insects corresponds to that found in vertebrates. Trujillo-Cenóz recognized in *Pholus* two categories of vesicles occurring in clusters within some of the axons of the neuropile: those with a "light" content which were found to be *c.* 200 Å in diameter, interspersed with a few larger profiles *c.* 600 Å in diameter, each with a dense content. Hess noted at least three groups of axoplasmic inclusions in *Periplaneta*, divided by him on the basis of size and appearance into "granules" (300–500 Å), "vesicles" (1000–1500 Å) and "droplets" (1200–2500 Å), the first of which predominated. In the *Periplaneta* material described by Treherne and Smith only two principal populations of vesicles were recognized: those with a lightly staining content, 300–500 Å in diameter, and others, usually with a distinct limiting membrane and an electron dense inclusion, 800–1100 Å in overall diameter: the distribution peaks of these two groups was found to be 450 Å and 1000 Å respectively (Figs. 24, 25). Many axon profiles in the neuropile are devoid of vesicles and contain only profiles of neurofilaments (Figs. 22, 24). In those profiles that contain vesicles, the smaller type may be present as a homogeneous population in concentrations of up to $4000/\mu^3$, or a varying admixture of the larger type may be present and, occasionally, the latter predominates.

Especially in the central region of the neuropile, then, large numbers of axon profiles are present, from several micra down to *c.* 0·15 μ in diameter, some containing vesicles, and others lacking them. The axon cell membranes are either very closely apposed or separated by gliocyte processes, and the question which must now be asked is whether all intimate axon contacts represent synaptic regions or whether any additional morphological indications exist whereby specific sites of synaptic activity within the neuropile may be recognized.

De Robertis (1955, 1956) and Palay (1958) described, in electron microscopic studies on the acoustic ganglion and cerebellar cortex of the rat, foci of electron dense material situated at intervals along the

pre- and sometimes also the postsynaptic membrane, backed by unusually closely packed clusters of synaptic vesicles within the presynaptic element. In accordance with the belief that the vesicles are

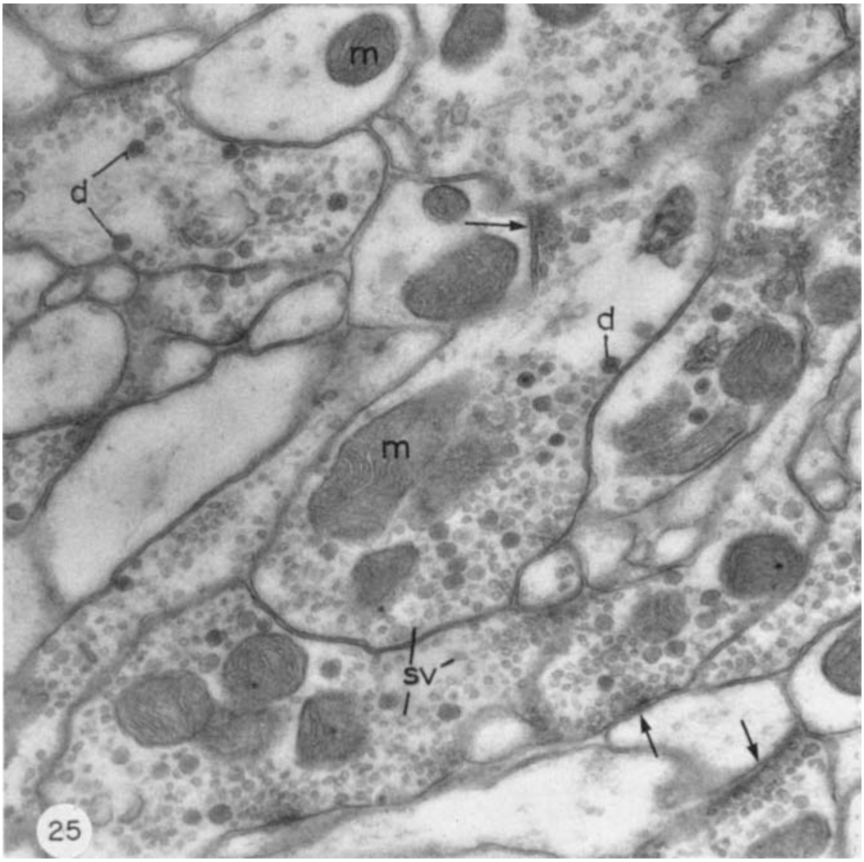

FIG. 25. A field within the neuropile in the last abdominal ganglion of *Periplaneta*. Profiles of axon branches of different sizes are present, and identifiable glial processes are in this instance virtually absent (cf. Fig. 22). Many of the axon profiles contain large numbers of synaptic vesicles *c*. 450 Å in diameter (sv) interspersed with larger profiles containing droplets of electron-dense material (d) possibly of neurosecretory products. The latter vesicles are *c*. 1000 Å in diameter, and are limited by a membrane. Mitochondria (m) are present in most of the axon profiles, but are more abundant on the vesicle-containing axoplasm. The synaptic vesicles are believed to be present in the presynaptic side of the synapse in the vertebrate central nervous system. Palay (1958) and de Robertis (1958) described local concentrations of these vesicles in regions of higher density along the presynaptic surface, believed by them to represent the synaptic sites. Similar associations are present in the insect neuropile: three such regions in this field are indicated with arrows, and this feature is seen to better advantage in Fig. 26. × 30,000.

situated in presynaptic axoplasm around the synaptic area, that they bear the chemical transmitter and that they initiate electrical activity in the postsynaptic element by discharging their content into the synaptic gap, Palay (1958) suggested that "the complex of a cluster of synaptic vesicles, associated with a focalized area of presynaptic

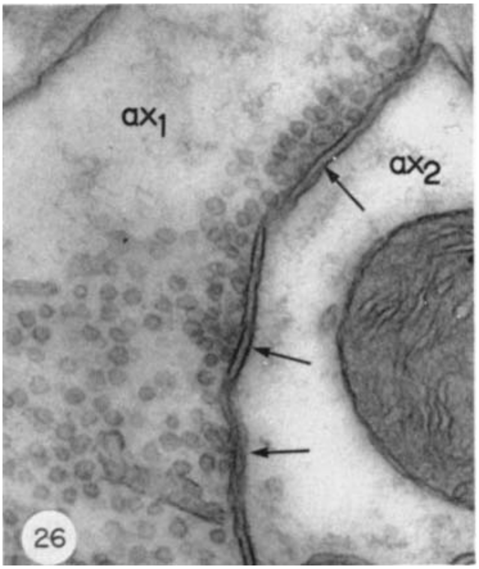

FIG. 26. The two axon profiles in this figure (ax_1, ax_2) are separated by an inter-cellular gap of *c.* 100 Å. The former contains large numbers of synaptic vesicles and these are concentrated at intervals along the inner surface of the axon plasma membrane in regions of higher density than elsewhere in the axoplasm: the distribution of these dense regions is "mirrored" in the apposed axon (ax_2) by foci of slightly increased density (arrows). It is believed, by analogy with the situation found in the vertebrate central nervous system (Palay, 1958; de Robertis, 1958) that these complexes represent active sites of synaptic contact between the vesicle-laden presynaptic axon terminal (ax_1) and the postsynaptic component (ax_2), mediated by a transmitter substance sequestered in the former, and liberated into the narrow gap between the two axon surfaces. × 55,000.

plasmalemma, and the synaptic cleft may be considered as a morphological subunit" of the synapse and that "these synaptic complexes may represent the actual sites of impulse transmission across the synapse".

Close examination of the complex organization of the insect neuropile reveals the presence of precisely similar structures (Figs. 25, 26) situated along the closely apposed membranes of pairs of axon profiles, one member of which contains synaptic vesicles while the other lacks these

structures. As described by de Robertis and Palay, these regions of high density are most evident in the vesicle-laden axoplasm; that is, by analogy with the vertebrate material, on the presynaptic side, and vesicles are similarly massed in the vicinity of the zones of density. The areas in which dense material is closely associated with the axons are of variable extent; their profiles are usually between 150 mμ and 500 mμ in length, and are thus similar in size to those described elsewhere. Trujillo-Cenóz (1959, 1962) pointed out that mere close contact between axon profiles in the neuropile, and the distribution of axoplasmic ("synaptic") vesicles, seems to be of too general occurrence to denote sites of synapse. The identification in the insect neuropile of the characteristic "synaptic foci" resembling those found in the vertebrate central nervous system may serve as a valuable pointer in a functional interpretation of the structural organization of this region of the insect ganglion.

Except for the "giant fibres", and the aggregation of very small axon profiles forming well defined tracts in the neuropile (which may, by inference from light microscopic studies, represent preterminal sensory components), there is at present no means of distinguishing the affinities of individual axon processes in the neuropile. Even in the more complex synaptic areas in the vertebrate central nervous system, on the other hand, knowledge of the general organization may permit the identification of individual profiles of nerve processes seen in the electron microscope. Thus in the neuropile surrounding the perikarya of the grey matter, the detailed organization of presynaptic components is the same as that in endings on the surface of the perikaryon, and hence a particular profile may be recognized as axonic or dendritic in origin (Palay, 1958). The synaptic areas of the insect neuropile presumably contain profiles of terminal arborizations of sensory, motor and internunciary components, present together in every field examined, and the distinctive topographical features of certain synaptic regions of the vertebrate nervous system are absent. Hess described longitudinal sections of axons in the neuropile of *Periplaneta*, with small vesicle-filled profiles believed to represent "end knob" terminations of fine axon branches, lying against or indenting the postsynaptic surface. In *Periplaneta*, Treherne and Smith found that the vesicle-containing axon profiles are usually between 0·15 μ and 4·0 μ in diameter, and that the presumed presynaptic terminals are often larger than the postsynaptic (Fig. 25). Occasionally a large axon seen in longitudinal section is found to contain synaptic vesicles aggregated along one side (Fig. 23), as described by Hess and Trujillo-Cenóz, perhaps indicating regions of

synapse with very small postsynaptic terminal branches. The occurrence of "cross contacts" and "longitudinal contacts" between larger axons, without any associated aggregation of synaptic vesicles in either axon, were described by Hess and Trujillo-Cenóz. This observation has been confirmed by Treherne and Smith. However, in view of the identification in the insect neuropile of specific associations between pairs of nerve fibres involving differentiated regions, described by de Robertis and Palay as synaptic sites, it seems preferable, at the present time, to adopt the hypothesis that synaptic transmission in the insect central nervous system may be mediated by a chemical transmitter, liberated from the vesicles contained within the axoplasm of the presynaptic member, and to reserve judgement on the significance of axon contacts within the neuropile in which neither member exhibits any characteristic axoplasmic differentiation.

The relegation of all synaptic contact to the neuropile, in insects, renders the structural analysis of this region a very difficult task. However, even from the fragmentary details at present available, it seems probable that the cytological features associated with regions of synaptic transmission in insects and vertebrates is essentially similar.

VI. The Extracellular System

A knowledge of the extent of the extracellular space within the insect nervous system is essential for any proper understanding of its physiology. It seems useful at this point, therefore, to survey the distribution

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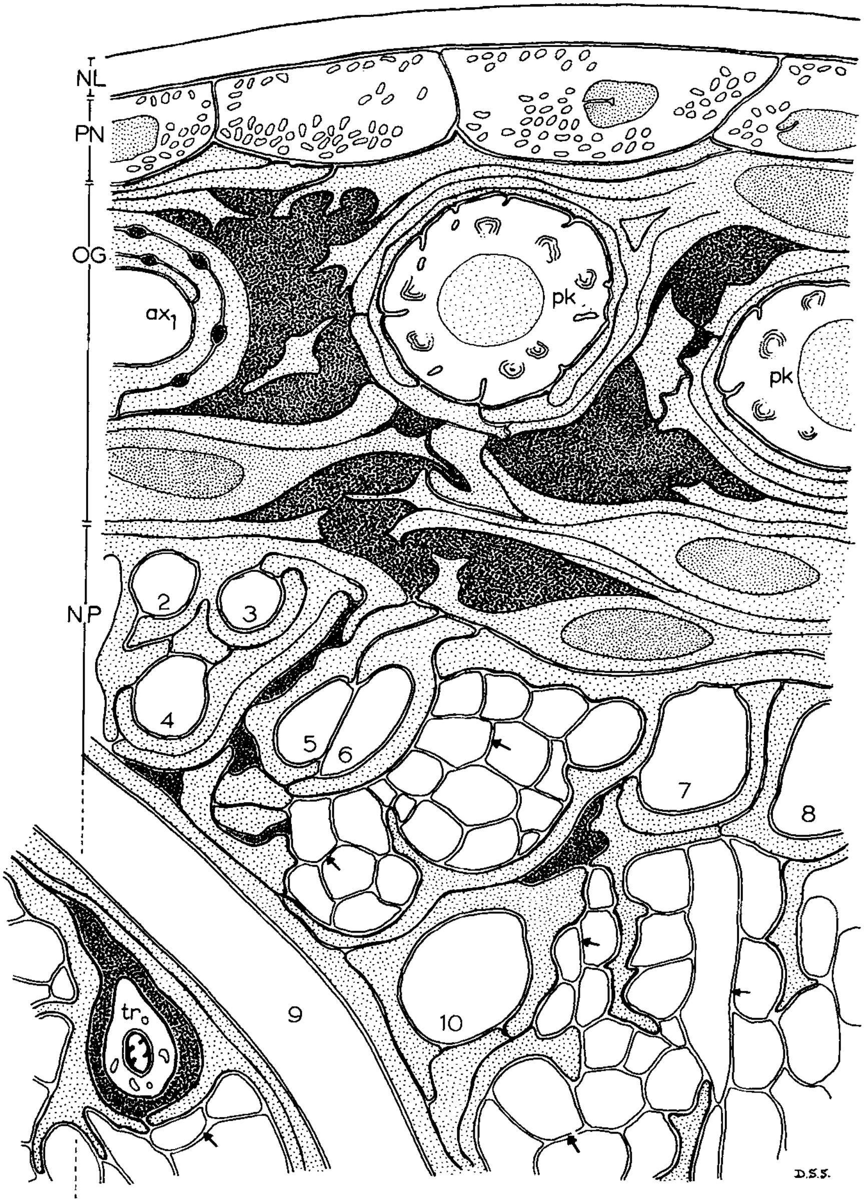
FIG. 27. Diagram illustrating the disposition of cellular and extracellular components in the last abdominal ganglion of *Periplaneta*. In this figure, glial cytoplasm is indicated by light stippling, and extensive extracellular spaces by dark stippling.

The ganglion is limited by a fibrous sheath, the neural lamella (NL), containing a collagen-like material, and beneath this is situated a cellular layer, the perineurium (PN). The outer layer of the ganglion (OG) between the perineurium and the neuropile (NP) contains the neurone cell-bodies or perikarya (pk) encapsulated by glial cell processes, while at ax_1 is seen a nerve fibre surrounded by a concentric glial sheath (cf. Fig. 14). The inner glial cells in this region of the ganglion send processes into the neuropile. The glial processes in the outer region of the ganglion form a loose meshwork defining more or less extensive extracellular spaces: the "glial lacunar system" of Wigglesworth.

The neuropile constitutes the synaptic region of the ganglion, and contains both nervous and glial elements. Many axon profiles within the neuropile are seen, in electron micrographs, to be ensheathed by glial cell processes (as in axons 2–10), while elsewhere, close apposition of axon plasma membranes is achieved by virtue of the absence of glial cell prolongations. In the latter instance, the axon surfaces are separated from each other by an extracellular gap of only *c.* 100–150 Å (arrows). In addition to this network of narrow channels between the axons, and between the axons and glial processes, larger extracellular spaces are occasionally present within the neuropile either surrounding the tracheoles (tr)



or lying between the glial processes (as between axons 4 and 5), and this neuropile extracellular system is confluent with the more extensive interglial cavities (the glial lacunar system) in the more peripheral regions of the ganglion.

Note that in this figure the width of the region between the perineurium and the neuropile has been reduced for purposes of clarity, while the size of the extracellular spaces between the axon branches and glial processes in the neuropile has been exaggerated.

of extracellular space in order to build up a composite picture of its occurrences, and to consider its functions in relation to the cellular organization of the insect nervous system.

The only detailed information dealing with the extracellular spaces in the insect nervous system appears to be that dealing with the central nervous system of *Periplaneta*. Figure 27 illustrates the extent of the extracellular space in the terminal abdominal ganglion of the cockroach, from information obtained by methods of light microscopy (Wigglesworth, 1960a) and electron microscopy (Treherne and Smith, in preparation). The most spacious extracellular component of the abdominal ganglion is that described by Wigglesworth as the "glial lacunar system", which has been recognized in electron micrographs as an irregular series of large cavities lying between the complex glial processes in the peripheral region of the ganglion. The larger tracheoles which enter the ganglion pass through this space before entering the neuropile, where they are separated from the axon and glial processes by a narrow extracellular channel, presumably confluent with the glial lacunae. In addition, extracellular channels seen as narrow spaces with periodic dilatations are interposed between the glial membranes of the mesaxon system, surrounding the sheathed axons lying outside the neuropile. A similar arrangement is found in association with larger axons within the neuropile, but in this case the glial sheaths may be extremely narrow. The dilatations appear to be characteristic of insect nerves and have been described, for example, in cockroach cercal nerve (Fig. 16), in smaller branches of motor nerves supplying the muscles (Fig. 32) (cf. Edwards *et al.*, 1958a) and in the abdominal connectives in *Periplaneta* (Fig. 12). In addition to the extracellular spaces described above, the neuropile is permeated by an extensive system of very fine channels, generally *c.* 100–200 Å in width. These include the spaces between the nervous and glial membranes and the interaxonal gaps.

The inulin space in the terminal abdominal ganglion of *Periplaneta*, measured by use of ^{14}C-labelled molecules, was found to be equivalent to 18·2 ± 1·7% of the total ganglion water (Treherne, 1962b). Since the finest channels in the neuropile have been shown to be of the order of 100–200 Å, and would presumably be accessible to inulin molecules (15 × 10 Å), it seems reasonable to assume that the extracellular system outlined above corresponds to the spaces occupied by the polysaccharide molecules. In the isolated abdominal nerve cord the rapidly exchanging tritiated water, which has been identified as an extracellular fraction, represents approximately 21·6% of the total water and is thus

equivalent to the space occupied by the inulin molecules (Treherne, 1962a, b).

The efflux of ^{24}Na from desheathed terminal abdominal ganglia and whole isolated abdominal nerve cords of *Periplaneta* was found to occur as a two-stage process with rapid and slowly exchanging ion fractions (Treherne, 1961e, 1962a, b) (Fig. 5). The rapidly exchanging fraction was identified with the ions contained in the extracellular spaces and was found to represent approximately a third of the exchangeable sodium in the ganglion. By inserting the value of 18·2% for the extracellular water, it was calculated that the concentrations of sodium ions in this fluid exceeded that of haemolymph by a factor of 1·8, and was two and a half times greater than that of the sodium contained in the cellular fraction of the ganglion. Further experiments using ^{24}Na, ^{22}Na, ^{42}K, ^{45}Ca and ^{36}Cl in whole isolated nerve cords, showed that the apparent concentrations of the rapidly exchanging fractions in the extracellular fluid were very different from those in the external medium (Table I).

TABLE I

Ionic concentrations in the rapidly exchanging extracellular fraction of the cockroach abdominal nerve cord expressed in terms of unit weight of tissue and concentration in extracellular fluid, relative to that of the external solution (Treherne, 1962a)

Ion	Concentration of rapidly exchanging fraction (mM/kgm tissue)	Estimated concentration in extracellular fluid (mM/l)	Concentration in external solution (mM/l)
Sodium	44·9 ± 2·3	283·6	157·0
Potassium	2·71 ± 0·15	17·1	12·3
Calcium	2·78 ± 0·12	17·6	4·5
Chloride	16·9 ± 1·5	106·7	184·1

The estimated values contained in Table I were shown to be consistent with the hypothesis that the ions were distributed between the extracellular fluid and the external medium according to a Donnan equilibrium. Thus the ratios for the monovalent cations and anions were found to be approximately equivalent:

$$\frac{Na_{out}+K_{out}}{Na_{extracellular}+K_{extracellular}} = \frac{157{\cdot}0+12{\cdot}3}{283{\cdot}6+17{\cdot}1} = 0{\cdot}56$$

and

$$\frac{\mathrm{Cl}_{\text{extracellular}}}{\mathrm{Cl}_{\text{out}}} = \frac{106{\cdot}7}{184{\cdot}1} = 0{\cdot}58$$

The higher concentration of calcium in the extracellular fluid relative to that in the external medium, as compared with the monovalent ions, is assumed to result from the enhanced Donnan effect due to the divalent charge of the ion. The expected distribution of calcium can be obtained by calculating the potential difference which would be developed between the extracellular fluid and the external medium for the steady state distribution of sodium, when:

$$E = \frac{RT}{F} \cdot \log_e \frac{\mathrm{Na}_{\text{extracellular}}}{\mathrm{Na}_{\text{out}}} = 58 \log_{10} \frac{283{\cdot}6}{157{\cdot}0} = 14{\cdot}9 \text{ mV}$$

where E is the potential difference, R is the gas constant, T is the absolute temperature and F is the Faraday. The value of 14·9 mV can be inserted in the following equation to obtain the ratio for the expected distribution of calcium ions:

$$\frac{\mathrm{Ca}_{\text{extracellular}}}{\mathrm{Ca}_{\text{out}}} = \mathrm{e}\,\frac{zFE}{RT} = 3{\cdot}5$$

where z is the valency of calcium. The calculated value of 3·5 is in reasonable agreement with the experimentally determined value of 3·8, which indicates that this divalent ion can also be considered to be distributed between the extracellular fluid and the outside medium according to a Donnan equilibrium.

The molecules containing the free anion groups which cause this Donnan effect in the extracellular spaces of the central nervous system have not been conclusively identified. In experiments in which ganglia were desheathed before being soaked in solutions containing ^{22}Na and ^{45}Ca it was shown that the concentrations of the ions fell to a lower level than would be expected on the basis of fixed anionic groups associated with structural elements in the extracellular spaces (Treherne, 1962b). It seems likely, therefore, that a significant portion of the cations in the extracellular fluid may be associated with some larger molecules in solution which are dispersed on desheathing the ganglion. The possibility that the free anionic groups may be those of collagen appears to be eliminated by the previously mentioned absence of collagen-like material from the extracellular spaces of the abdominal ganglia of *Periplaneta*. It can be calculated, for example, using the figure of 77·2 free anion equivalents/10^5 g protein (Tristram, 1953), that

if all the anionic groups were available then the collagen required to produce the demonstrated ionic distribution would represent at least 5% of the wet weight of the nerve cord. Collagen-like material has, however, been identified in the extracellular spaces of the auditory ganglion of *Locusta migratoria* (Gray, 1959) so that it is possible that this could contribute to a Donnan effect in portions of the nervous system of other species.

The possibility remains that the anion groups in the extracellular spaces of the cockroach central nervous system could be those associated with the acid mucopolysaccharide demonstrated in the ganglia of *P. americana* by Ashhurst (1961c). By analogy with the polysaccharide component of the mucoprotein in cartilage, studied by Kantor and Schubert (1957), it might be supposed that a substantial proportion of anionic groups might be free and associated with inorganic cations in this substance in the cockroach central nervous system. The distribution of free anionic groups in the complex channels and lacunae, which form the extracellular system, is not known. It is conceivable, for example, that local differences in concentration of cations may be maintained by differential distributions of large molecules containing free anionic groups.

The demonstration of the presence of the Donnan equilibrium between the extracellular fluid and the haemolymph provides a possible explanation for the effects of removal of the sheath surrounding the central nerve system of *Periplaneta* recorded by Twarog and Roeder (1956). It is clear that removal of the nerve sheath results in some very dramatic changes in the composition of the extracellular fluid, due to the disruption of the Donnan equilibrium. As a result of desheathing, the concentration of cations will fall and that of the diffusible anions will tend to rise to levels close to those in the outside medium. By analogy with vertebrate fibres (Lundberg, 1951; Stämpfli and Nishie, 1956) it might be expected that these ionic changes would affect the rate of depolarization in conditions of high external potassium (Treherne, 1962a, b). Thus the enhanced rates of depolarization demonstrated in the absence of the nerve sheath in this insect (Fig. 7) might be a reflection of the changed ionic environment in the extracellular spaces, rather than to any properties of the nerve sheath as an appreciable diffusion barrier. Some recent experiments have shown, in fact, that the rate of decrease of the compound action potential in desheathed preparations occurred more slowly, in conditions of high external potassium, when the concentrations of other ions were elevated to approximate to the composition of the extracellular fluid under these conditions (Fig. 28)

(Treherne, 1962c). This effect appeared to be due to increased concentration of the extracellular sodium rather than the calcium ions, and at an external concentration of 70 mM/l K^+ occurred at approximately half the rate of depolarization of the intact preparation. In experiments in which the sodium and potassium concentrations were reversed (i.e. 156 mM/l K^+; 12·3 mM/l Na^+) the very low sodium level produced a rather smaller effect on the rate of decline of the compound action

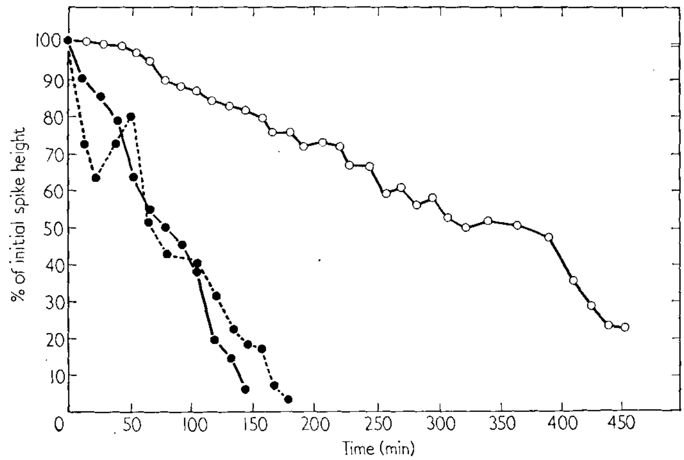


FIG. 28. Comparison of the rate of loss of conduction across the desheathed 4th abdominal ganglion of *Periplaneta* when perfused with a high potassium solution (70 mM K^+; 99·3 mM Na^+; 4·5 Ca^{++}—closed circles) and with a solution containing elevated cation concentrations (open circles) corresponding to the levels in the extracellular fluid of a perfused intact ganglion (i.e. 85·9 mM K^+; 153·0 mM Na^+; 17·1 mM Ca^{++}). These observations were carried out on the same preparation which was washed with normal, physiological solution between each perfusion to restore the original electrical activity. The preparation was first perfused with the 70 mM K^+ (continuous line), then with the "extracellular solution" and once again with the 70 mM K^+ solution (broken line) (Treherne, 1962c).

potential. Elevation of the cation concentrations to the extracellular level appeared to have little effect on the rate of decrease of synaptic transmission across the desheathed last abdominal ganglion when this was irrigated with solutions containing massive concentrations of acetylcholine ($5{\cdot}0 \times 10^{-2}$ moles).

It seems clear from the above evidence that the changes in the level of the extracellular cations, caused by the disruption of the Donnan equilibrium, only partly accounted for the enhanced rates of depolarization obtained on removal of the perilemma in solutions containing high

concentrations of potassium ions. Of course, the possibility exists that some unidentified factor may have been lost from the extracellular fluid as a result of the desheathing procedure. It also seems likely, however, that the electro-physiological effects obtained on desheathing may in part result from the nature of the organization of the extracellular system in the abdominal ganglia. It has already been shown that the extracellular system in the terminal abdominal ganglion of *P. americana* consists of large glial lacunar spaces towards the periphery, which account for the greater part of the extracellular space, together with a ramifying system of exceedingly fine channels in the deeper layers of the ganglion. It thus seems possible that the rapid exchanges of labelled ions and molecules which have been shown to occur across the nerve sheath are, in fact, those taking place between the haemolymph and the large peripheral extracellular spaces. The relatively large rapid exchanges of this kind might effectively obscure any slower exchanges taking place between the large peripheral spaces and the very restricted channels in the deeper portions of the ganglion. Removal of the nerve sheath is known to result in an increase in the measured inulin space (Treherne, 1962b). The possibility thus exists that the desheathing of portions of the abdominal nerve cord results in an increased accessibility of ions and molecules to the nerve cells due to some increases in the extracellular spaces in the central portion of the ganglion. This factor, together with the ionic changes produced in the extracellular fluid, could therefore account for a substantial proportion of the rapid depolarization obtained on desheathing insect nerves in high concentrations of potassium ions, rather than to any properties of the nerve sheath as a signified diffusion barrier.

A certain amount of information is available on the factors involved in the movement of ions and molecules in the extracellular spaces of the cockroach central nervous system. It has been shown, for example, that the ratio of the rates of efflux of ^{14}C-inulin molecules and ^{24}Na ions was similar to that for their free diffusion constants (Treherne, 1961e). These results were interpreted as showing that the movements of the polysaccharide molecules were relatively unrestricted in the extracellular spaces of the abdominal nerve cord of this insect. That the diffusion of such a large molecule as inulin (15 $\times$ 30 Å) should apparently not be restricted was unexpected, for it is known that the movements of even such small molecules as urea (1·6 Å) and sucrose (4·4 Å) are significantly reduced in collodion membranes with effective pore radii of 150 Å (Manegold, 1929). An apparently free diffusion of inulin would thus imply the existence of a *continuous* system of relatively large

extracellular channels. More recent investigations have shown, however, that the similarity of the ratio for the diffusion of sodium ions and polysaccharide molecules in extracellular spaces to that of their free diffusion constants, was due to the slowing down of movements of positively charged ions in these spaces (Treherne, 1962b). These results show that the inulin molecules were, in fact, diffusing in these spaces much more slowly than would be expected on the basis of their free diffusion constants. Such restricted diffusion of these polysaccharide molecules may be related to movement through extracellular channels of the dimensions demonstrated in electron micrographs of the cockroach terminal abdominal ganglion. The factors limiting the efflux of positively charged ions from the extracellular spaces have not been identified. Such an effect could result, for example, from a peripherally situated cation barrier, or from the attraction provided by free anion groups encountered in the passage of these ions through the extracellular spaces of the abdominal nerve cord.

The architecture of the extracellular system in the region of the axons is thought to affect profoundly the electrical behaviour of these structures. In cockroach giant axons, as in those of the squid (Hodgkin and Keynes, 1955; Frankenhaeuser and Hodgkin, 1956), the positive phase of the action potential has been postulated to result from a delayed and sustained increase in potassium conductance (Yamasaki and Narahashi,1959). The positive phase in cockroach giant axons was found to decrease at elevated potassium levels, and it was suggested that the decrease in the positive phase during a train of impulses was caused by an accumulation of potassium ions, released during the latter portion of each action potential, at the surface of the axon (Narahashi and Yamasaki, 1960). In the squid axon (Frankenhaeuser and Hodgkin, 1956), it has been postulated that the negative after-potential could result from an external aqueous layer of the order of size (100 Å) of that contained between the surface of the axon and the inner border of the Schwann cell shown in the electron micrographs of Geren and Schmitt (1954). Narahashi and Yamasaki have calculated that the increase in the potassium concentration at the surface of the cockroach axon immediately after the spike averages 2·0 mM. Using the figure of $3{\cdot}6 \times 10^{-12}$ moles K^+/cm^2/impulse for the release of potassium ions from the squid axon (Keynes and Lewis, 1951) then it can be calculated that the volume of the aqueous layer at the axon surface would be

$$\frac{3{\cdot}6 \times 10^{-12}}{2{\cdot}0 \times 10^{-3}} = 1{\cdot}8 \times 10^{-9}\ \mathrm{l/cm^2}$$

which is equivalent to a thickness of 180 Å. This value falls within the range of the dimensions of 100–200 Å found for the gap between the axon and glial membranes in electron micrographs of the cockroach central nervous system (Treherne and Smith, in preparation). Thus if it is assumed that the potassium released from unit surface area in the cockroach axon at each impulse is of the same order as that of the squid, then it can be postulated that the negative after-potential in the insect axon could result from the accumulation of the ions in the spaces shown in electron micrographs.

The apparent extracellular spaces in the region of the excitable membrane in the cockroach central nervous system has been shown to consist of a system of concentric channels which is confluent with the 100–200 Å gap adjacent to the axon surface (Fig. 29). The accumulation of potassium in this gap implies that the movement of ions along the concentric channels must be a limiting factor in their dispersal from the excitable membrane. Now the time course for the delay of the negative after-potential in cockroach giant axons was found to follow a simple exponential decline with a mean time constant of 9·2 msec (Fig. 30) (Narahashi and Yamasaki, 1960). In the squid giant axon the equivalent time constant was between 30 and 100 msec (Frankenhaeuser and Hodgkin, 1956), and it was suggested that the external barrier surrounding the cockroach axon was less effective in restraining the potassium ions than that of the cephalopod. It has already been mentioned that one unusual feature of the concentric channels in the cockroach is the presence of a series of dilatations (Fig. 14). It is conceivable that these dilatations could act as reservoirs which, by increasing the effective volume of the extracellular fluid in the region of the axon surface, could facilitate the dispersal of potassium ions from the excitable membrane. It is possible, therefore, that the presence of such dilatations could contribute towards the relatively short time constant for the decline of the negative after-potential in the cockroach axons.

The investigation of Narahashi and Yamasaki (1960) showed that the time course of the decay of the negative after-potential following repetitive stimulation was not a simple exponential process (Fig. 31). These authors attributed the slow, second phase of the decay to the presence of the nerve sheath which they supposed would restrain an appreciable proportion of the excess potassium released by repetitive stimulation. The possibility also exists, however, that this form of the decay of the negative after-potential could be due in part at least to the presence of the enlarged spaces in the extracellular channels surround-

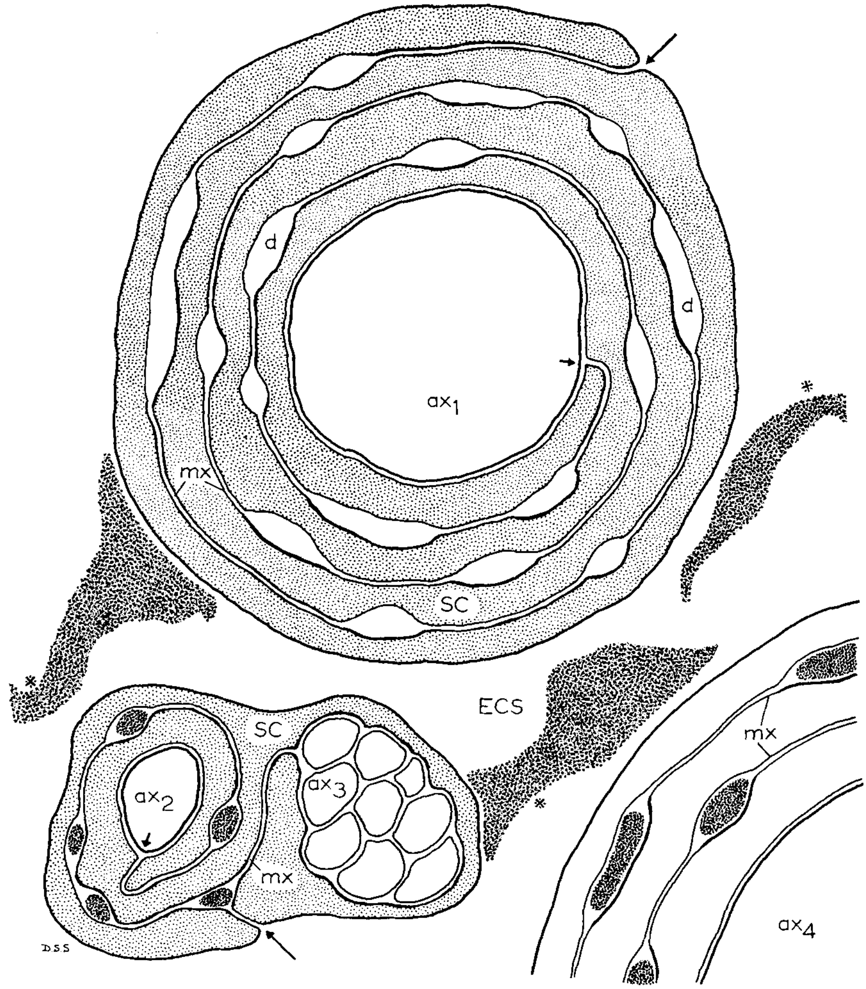


FIG. 29. Diagram illustrating the relationship between axons and glial cell sheaths in the central nervous system of *Periplaneta*. The larger axons (as at ax_1) are invested with a concentric Schwann cell sheath (SC) (or "lemnoblast") within which a mesaxon invagination extends from the surface (long arrow), in a more or less spiral course, and terminates as a sheath separated from the plasma membrane of the axon by a gap of *c.* 100–150 Å; the membranes of the mesaxon fold diverging at the point indicated by a short arrow. The mesaxon folds (mx) thus define an extracellular channel, *c.* 100–150 Å in width, at intervals along which are interposed wider dilatations or lacunae (d). The latter usually contain profiles of a homogeneous extracellular material, as is indicated in the sheaths surrounding the axons at ax_{2-4}. The smaller axons may be associated with a separate mesaxon invagination, as between the long and short arrows at ax_2, or a group of axons may be surrounded by a common Schwann cell process as at ax_3.

In the smaller peripheral nerves, one or more axons may be present; the Schwann cell or lemnoblast may be invaginated to form mesaxons at several points, and the mesaxons may branch and rejoin along their course (Edwards *et al.*, 1958a). In the large cercal nerve, many axons of varying size are present, and the axon sheaths are closely applied to each other. In the outer regions of the ganglion, however, the sheathed axons may be separated by more extensive extracellular spaces (ECS; cf. Fig. 13) of the "glial lacunar system". Such spaces often contain sheets of extracellular homogeneous material (*) similar in appearance to the deposits occurring within the dilatations of the mesaxon system.

ing the surface of the axon. Thus, stimulation at 50 impulses/sec might be expected partially to fill the spaces with released potassium ions. At the termination of stimulation the ions immediately in contact with the excitable surface, where they would be at maximal concentration, might diffuse away rapidly until they achieved an equilibrium with those in the enlarged spaces. This initial rapid fall in potassium concentration in the surface of the axon could thus produce the rapid portion of the

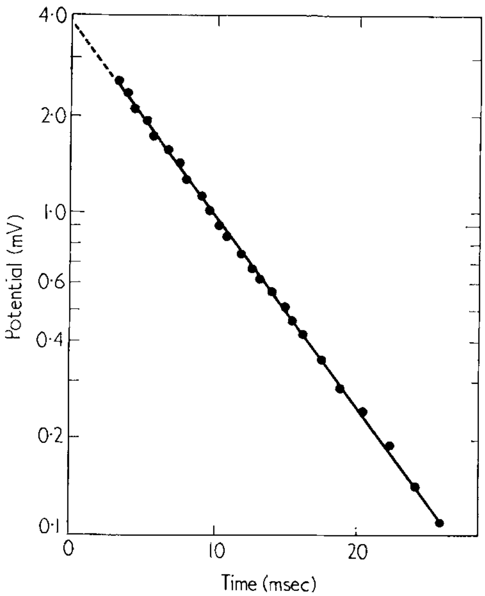


FIG. 30. The time course for the decay of the negative after-potential following a single impulse recorded from a cockroach axon bathed in normal Ringer's solution (Narahashi and Yamasaki, 1960).

decay illustrated in Fig. 31. The subsequent slow decay following stimulation at 50 impulses/sec would result, according to this hypothesis, from the relatively slow fall in the potassium level as the reservoirs of ions in the large spaces disperse into other parts of the extracellular system. The reduction of the rapid component of the decay of the negative after-potential following a train of impulses at 200/sec (Fig. 31) could be due to an initial higher level of potassium ions in the reservoirs, resulting from the more rapid stimulation. It follows from this interpretation that at even higher rates of stimulation it should be possible to obtain a situation in which the initial concentration of potassium

ions in the dilatation reservoirs would approach that in the fluid in contact with the axon surface, so that the greater part of the decay of the potential might be expected to occur at a slow rate.

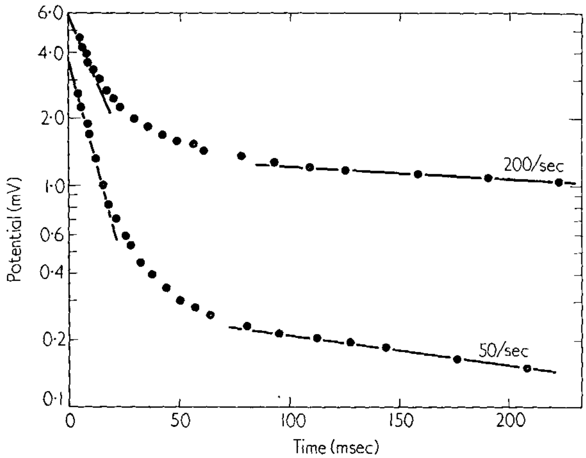


FIG. 31. The time course for the decline of the negative after-potential in a cockroach axon following a train of impulses at 50 and 200/sec (Narahashi and Yamasaki, 1960).

VII. THE NEUROMUSCULAR JUNCTION

At the neuromuscular junction, an association is established between the termination of one or more motor axons and the surface of the muscle cell, across which a signal received from the central nervous system may be transmitted, ultimately eliciting a response from the contractile mechanism of the fibre. Neuromuscular junctions and synapses elsewhere in the nervous system have many morphological and physiological features in common, and the present aim is to give some account of the cytological structure of the insect neuromuscular junction and to compare this with its counterpart in vertebrate muscles and with central nervous synapses.

Light microscopic studies showed that the "motor nerve ending" on striated muscle is anatomically a polymorphic structure: in many vertebrate muscles a single complex "end-plate" is present on each fibre, while in insects, for example, the terminal branches of axons may contact the fibre in many places, either in association with conical projections (Doyère's cones) or as a ramifying system deployed over the fibre surface. However, the electron microscope has confirmed the

supposition that variation at this level masks an underlying structural similarity between one type and another.

The vertebrate motor end-plate has been most extensively studied in the light and electron microscope, and will be used here as a reference point with which to compare and contrast the corresponding regions of insect muscles. With regard to the former, morphological, cytochemical and cytological details and references may be found in the accounts of Andersson-Cedergren (1959) and Couteaux (1958, 1960). As in the case of central nervous synapses, the electron microscope confirmed the generally held belief that there is no cytoplasmic continuity between the excitable cells at the nerve-muscle junction; in each instance, the pre- and postsynaptic surfaces are separated by a narrow gap devoid of cellular intrusions. The myelin sheath that accompanies the vertebrate nerve fibre along its peripheral course from the central nervous system is lost just before the surface of the fibre is reached, and the terminal branches of the axon continue on in association with specialized "teloglial" cells. The axon branches are accommodated in "synaptic gutters"; indentations of the fibre surface, and the teloglial processes are, at this region, restricted to "caps" overlying the axon and do not obstruct the close apposition between the inner surface of the latter and the membrane of the muscle fibre. The membrane of the synaptic gutter is folded in a complex manner beneath the axon, and the "junctional folds" thus formed, together with the space of 400–500 Å separating pre- and postsynaptic membranes, is largely filled with a homogeneous extracellular material resembling that of the basement membrane. Several synaptic gutters, each containing a terminal axon branch, constitute the end-plate, and the arrangement of these affords the branching or palmate appearance of the ending in light microscopic preparations. The most striking feature of the axoplasm of the terminal branches is the presence of large numbers of profiles similar in size and appearance to the "synaptic vesicles" of the central nervous system: these were first described by Palade (1954), and subsequently in other vertebrate myoneural junctions by Robertson (1956), Reger (1959, 1961), Andersson-Cedergren (1959), Birks *et al.* (1960) and others. The cytoplasm of the terminating axon contains many mitochondria; a feature that is also found in most presynaptic terminals in the central nervous system.

The cytological organization of neuromuscular junctions in insects has been investigated by Edwards *et al.* (1958a, b) in femoral muscle of *Vespula carolina* and in flight and tymbal muscles of *Tibicen linnei*; by Smith (1960, 1961b) in flight muscle of *Tenebrio molitor* and *Aeshna*

sp.; by Edwards (1959) in intersegmental muscle of *Blatta germanica*, and by Edwards (1960) in a thoracic (non-flight) muscle of a dragon-fly; while preliminary observations have been carried out by Smith (unpublished) on coxal and indirect flight muscle of *Apis mellifera*. The organization of the peripheral nerves supplying the muscles conforms to the "tunicated" arrangement first described by Edwards *et al.* (1958b): no myelin is present, but the surface membranes of the ensheathing glial cells (lemnoblasts or Schwann cells) are invaginated to form a more or less concentric or branching mesaxon system, similar in its general organization to that enveloping the axon trunks lying outside the neuropile in the central ganglia, in the cercal nerves and in the connectives (Hess, 1958a, b; Treherne and Smith, in preparation). The dilatations of "lacunae" occurring at intervals along the mesaxon folds (Edwards *et al.*, 1958a, b; Smith, 1960; Treherne and Smith, in preparation) are irregular and often of considerable size (Fig. 32). All authors agree that the lemnoblasts of the peripheral nerves are covered with a layer of basement membrane, within which tracheolar cell processes are often embedded. This layer does not appear to contain the collagen-like fibrils present in the neural lamella of the ganglia.

Before the cytological features of the insect neuromuscular junction are described, it should be pointed out that while vertebrate "fast" fibres receive the branches of a single axon at a single end-plate, as described above, insect and other fibres may not only bear several junctions, but may also be supplied with branches of more than one nerve, each eliciting a different type of response from the fibre: the physiological significance of such multiple innervation has been reviewed by Hoyle (1957). It is sufficient for the present to note that while certain insect muscles are innervated by "slow" and "fast" fibres (Pringle, 1939; Hoyle, 1957; Cerf *et al.*, 1959), innervation of non-fibrillar flight muscle, of fibrillar flight muscle (other than of the beetle *Oryctes*) (refs in Darwin and Pringle, 1959), and of cicada tymbal muscle (Pringle, 1957) appears to be uniformly of the "fast" non-facilitating type. In a cytological context, it should be pointed out that the "axon" of the physiologist may relate to a single nerve branch, actually containing more than one slender axon process, enclosed within a common sheath. There is no reason to suppose, however, that the axons belonging to the same nerve branch are physiologically heterogeneous.

The cellular arrangement of the insect neuromuscular junction corresponds to that of the vertebrate motor end-plate in that close

apposition of plasma membranes of axon branch and muscle fibre is achieved. In all the insect muscles mentioned above, the basement membrane of the glial sheath coalesces with that of the sarcolemma before the junction is reached (Fig. 32) and, except in flight muscle of *Tenebrio* and *Aeshna* (among the instances so far studied), the terminal glial cell layer corresponding to the vertebrate teloglia is retained as an

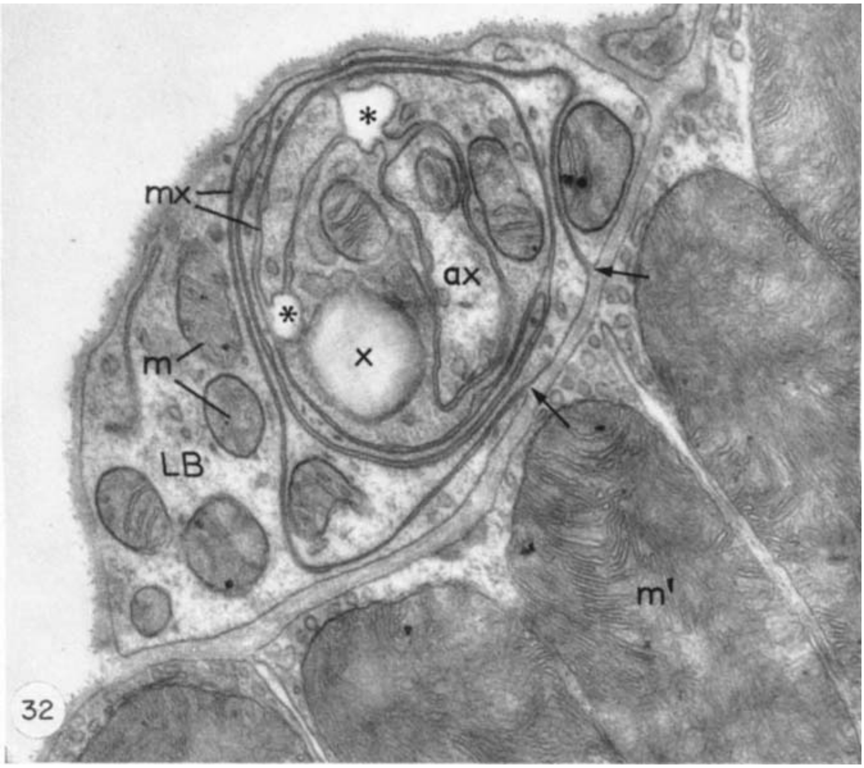


FIG. 32. A presynaptic motor nerve branch associated with the surface of an indirect flight muscle fibre of *Apis* (Smith, unpublished). The axon ax is surrounded by a lemnoblast (glial) cell sheath (LB). Note the two mesaxons originating as invaginations from the superficial lemnoblast plasma membrane (arrows). The mesaxon folds (mx) follow a branching course around the axon (the "tunicated" arrangement) and the mesaxon membranes separate periodically to establish dilatations or lacunae (*) before the innermost mesaxon fold diverges to accommodate the axon (Edwards *et al.*, 1958a). Note the small mitochondria in the lemnoblast (m) and the very large mitochondria (sarcosomes) within the muscle fibre (m′). The circular profile at x is of unknown significance, but does not appear to be continuous with the mesaxon lacunar system. Small vesicles are present just beneath the muscle plasma membrane in all regions of the fibre, in this instance, and do not appear to be associated in any way with the neuromuscular junction. ×36,000.

outer covering over the synapsing axon terminal (Figs. 34, 35). The inner surface of the latter approaches even more closely to the muscle plasma membrane than in the vertebrate ending, by virtue of the absence of extracellular basement membrane material from the synaptic gap,

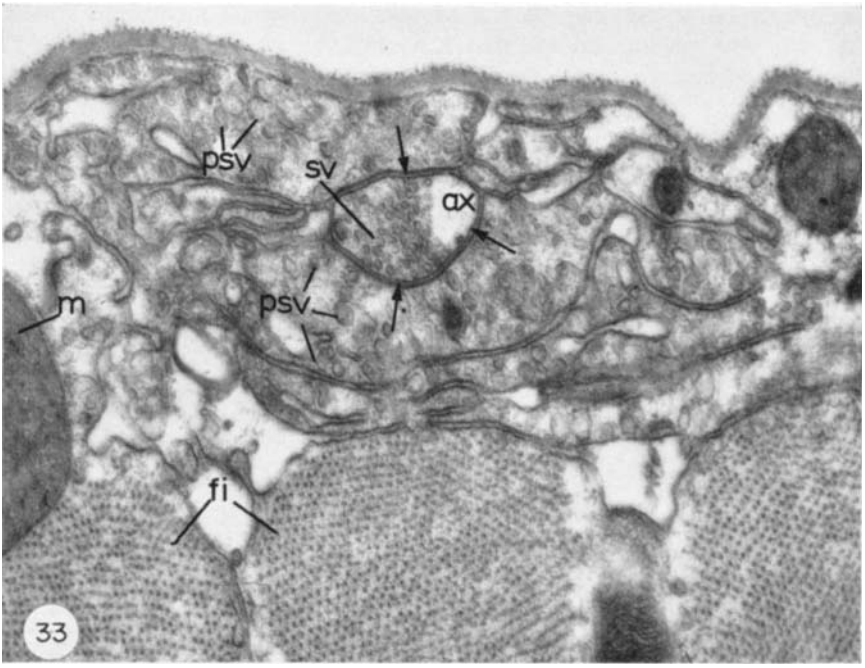


FIG. 33. A neuromuscular junction on an indirect flight muscle fibre of the beetle *Tenebrio* (from Smith, 1960). Whereas in other instances the terminating axon is still "capped" by the lemnoblast (cf. Fig. 35), this covering is in this case lost completely in the junctional region, and the axon ax makes a circumferential synapse with the muscle fibre into which it is invaginated (arrows). The postsynaptic region of the fibre contains large numbers of postsynaptic vesicles (psv) within a complex system of muscle plasma membrane folds. The concentration of these vesicles is characteristic of the junctional region in this instance, and these are larger than the synaptic vesicles (sv) within the terminal axoplasm. Note the myofibrils (fi) and a large mitochondrion (sarcosome) within the fibre (m). × 30,000.

which is thereby reduced in width to *c*. 100 Å. The nerve terminals in *Tenebrio* and *Aeshna* flight muscles differ from the above in that the lemnoblast is ultimately completely discarded, and the naked axon either lies in a groove (*Aeshna*) or is fully invaginated (*Tenebrio*) into the fibre surface, forming, in the latter instance, a circumferential synapse (Fig. 33).

In the neuromuscular junctions of *Vespula*, *Tibicen*, *Aeshna* and *Tenebrio* it has been found that the junctions are effected by single axons or axon branches spread diffusely over the fibre: in *Tenebrio* flight muscle, for example, it was found that any transverse section of a single fibre will pass through several junctional profiles; attesting to the richness of the neuromuscular supply. Myoneural junctions of the aggregated multiaxonal type have been described in *Blatta* intersegmental

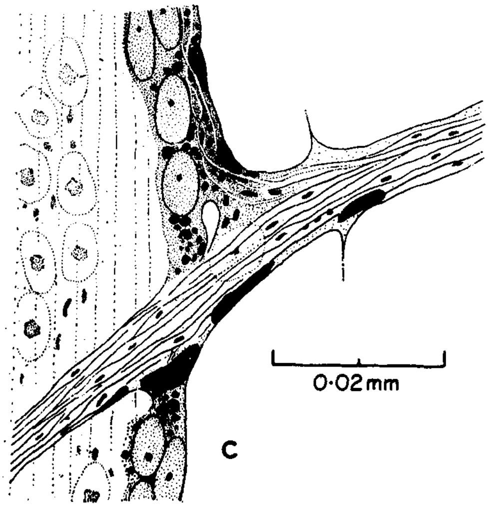


FIG. 34. Transverse nerve giving off branches to ventral abdominal muscle in 4th-stage larva of *Rhodnius prolixus* (osmium/ethyl gallate preparation). The nuclei of the nerve sheath have solid outlines while those of the muscle are represented in dotted outline. Note mitochondria in the axons and in the nerve sheath and the large, darkly stained elongated inclusions in the nerve sheath. The latter inclusions have not been identified in electron micrographs of *Periplaneta americana* (Wigglesworth, 1959a).

muscle (Edwards, 1959), in a longitudinal thoracic muscle (not a direct flight muscle) (Edwards, 1960) and in *Apis* coxal muscle (Smith, unpublished). In these instances more than one axon is involved in each motor ending. In *Blatta*, Edwards (1959) found that each of several motor endings on a single fibre involves up to three nerve branches, each containing numerous axons, and such endings presumably represent the "Doyère's cone", while multiterminal endings each involving a single axon or axon branch represent the diffuse filiform system recognized in the light microscope.

The cytological organization of a multiaxonal nerve ending on *Apis* coxal muscle is illustrated in Figs. 35, 36, and these electron micrographs

may be compared with the drawing of a similar ending in *Rhodnius* abdominal muscle (Wigglesworth, 1959a; Fig. 34). Wigglesworth noted that the nerve contacts the fibre periodically, and that axons destined for synapse at each junction are associated with a glial sheath containing nuclei and numerous small mitochondria. In Figs. 35 and 36 profiles of several axons are seen contacting the muscle fibre surface at the points indicated: a presynaptic axon is included in this field and small mitochondria, such as are found in the glial sheath of this axon, are found in large numbers in the glial cytoplasm overlying the nearby synapsing axon profiles. The glial cells covering the outer surface of the synapsing axons or axon branches appear to form a complex interdigitating association with processes of the muscle cell membrane, and extracellular spaces lined with basement material are interpolated between them.

Although, as Edwards (1959, 1960) suggests, multiaxonal junctions may well involve both "slow" and "fast" axons, there is at present no known cytological feature whereby these may be distinguished. However, electron microscopic examination of the axons of nerve branches demonstrably "slow" or "fast" in relation to the muscle fibre should be a feasible task, and such analysis might well indicate synaptic distinctions as yet unrecognized.

All electron microscopic investigations of insect neuromuscular junctions have shown that vesicles and mitochondria are massed in the

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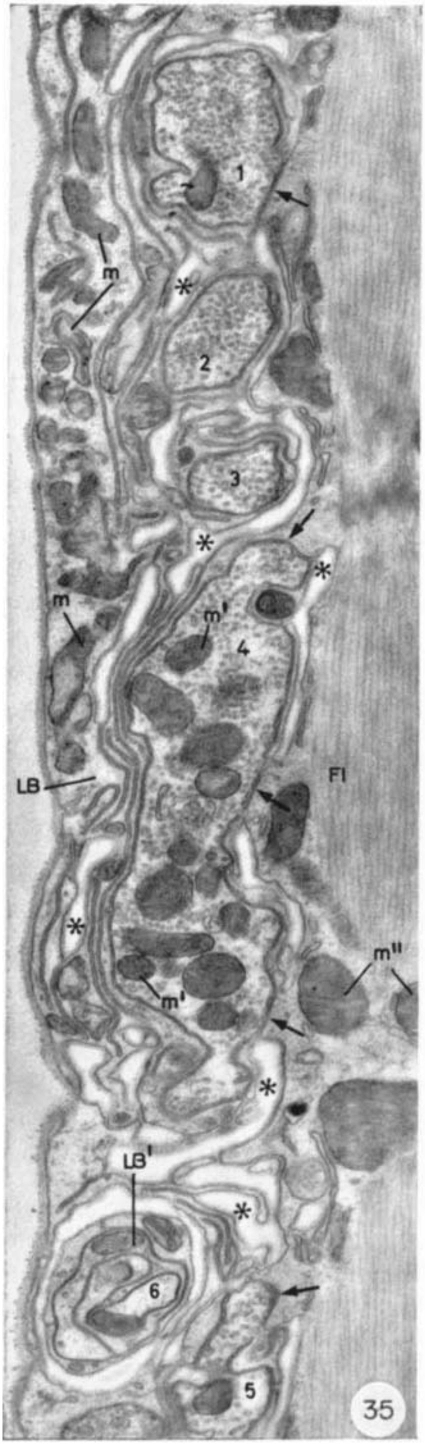
FIG. 35. A low-power survey electron micrograph of a motor nerve terminal on a coxal muscle fibre of the bee, *Apis*. Six axon profiles are present (1–6): the first five of these contain large numbers of synaptic vesicles, and in several places (arrows) the glial ("lemnoblast") sheath elsewhere covering the axons, is absent, allowing a close apposition of the axon and muscle fibre plasma membranes (see Fig. 36). The axon profile at 6 is still surrounded by a complete lemnoblast sheath (LB′), and the axoplasm here lacks synaptic vesicles. Profiles 2 and 3 appear to be closer to their termination; they contain vesicles, but are still surrounded by a tenuous glial sheath.

The axon profiles are "capped" by a continuous lemnoblast sheet (LB) (cf. Fig. 31) corresponding to the teloglia of the vertebrate motor end-plate. The lemnoblast cytoplasm contains many mitochondria (m), smaller than those within the terminal axoplasm (m′) or within the muscle fibre (m″). The lemnoblast covering at the ending forms a complex interdigitating system of processes above the axon profiles, and extracellular gaps (*), lined with basement membrane material, are situated between the processes and between these and the surface of the fibre. A portion of a myofibril lying beneath the nerve terminal is seen at FI.

It should be noted that these axon profiles may represent separate terminating axons, or branches of a single axon. ×19,000.

FIG. 36. An enlargement of a portion of the last figure. Note the concentrations of synaptic vesicles within the terminal axoplasm (sv). The basement membrane material overlying the muscle plasma membrane (bmm) and lemnoblast processes (bml) appears to be absent from the regions of synaptic contact, at which the plasma membranes of axon and muscle fibre are separated by a gap of only *c*. 100 Å (arrows). ×33,000.


1
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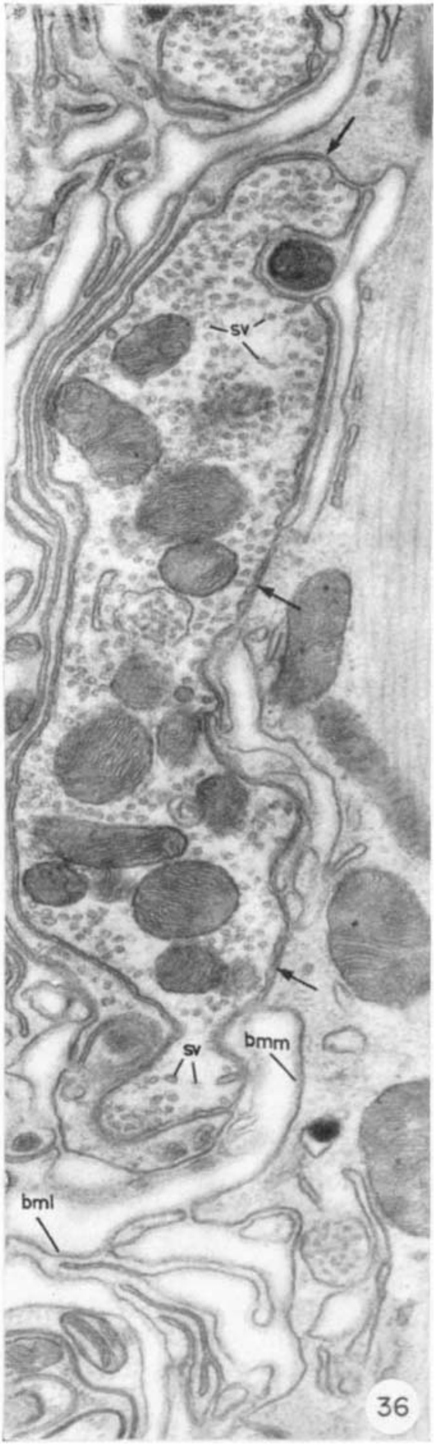
sv
sv
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36

terminal axoplasm, precisely as in the vertebrate motor end-plate (Figs. 33, 35, 36). The organization of the sarcoplasm immediately underlying the junction appears to be more variable. In vertebrates, Reger (1958) and Andersson-Cedergren (1959) described a rather sparse distribution of vesicles concentrated in, but not confined to, the "sole plasm" region surrounding the junctional folds, while in another instance (Reger, 1961) such vesicles are absent and the post-synaptic area contains groups of small granules. *Tenebrio* flight muscle was found to be unusual, in that the region surrounding the axon terminal contains complex folds of the muscle cell membrane containing many vesicles, larger than those within the axoplasm (Smith, 1960). Edwards *et al.* (1958b) described a well-developed "rete synapticum" of membrane profiles in this region of cicada flight muscle fibres, which they considered to be an elaboration of the sarcoplasmic reticulum. A more restricted zone containing small numbers of granules and vesicles occurs in the sarcoplasm adjoining the synapsing axon in the other insect muscles investigated (Edwards *et al.*, 1958a; Edwards, 1959).

In short, it appears that there are no specific cell components or grouping of components that are characteristic of the postsynaptic region of the muscle fibre, and it is only the organization of the terminal axoplasm and the essential features of the apposition of the synaptic surfaces that are held in common by all insect and vertebrate neuromuscular junctions thus far examined. Recent ideas concerning the possible functional significance of the organization of the neuromuscular junction have been formed primarily with reference to vertebrate muscle fibres, and it is interesting therefore to consider the peculiarities of neuromuscular physiology in insects in the light of the observed similarities between motor nerve endings in vertebrate and insect muscles.

In the fast fibres of vertebrate muscles, the arrival of a motor impulse at the nerve ending produces an "end-plate potential" in the end-plate region of the fibre surface (Feng, 1941), and when this local depolarization is of sufficient size, it initiates a quite distinct membrane response—a propagated action potential spreading without decrement over the fibre surface, followed after a short delay by a rapid "twitch" response. The release of acetylcholine from the nerve terminal was first demonstrated by Dale *et al.* (1936) and Brown *et al.* (1936), and subsequent neurophysiological investigations have indicated that the rôle of this substance, in vertebrate muscle, is to establish the end-plate potential, by altering the ionic permeability of the muscle cell membrane under-

lying the nerve ending (refs. in Katz, 1962). The presence in the end-plate region of large concentrations of a cholinesterase, capable of hydrolysing the acetylcholine secreted by the axon terminal, was demonstrated histochemically by Koelle and Friedenwald (1949), and the postsynaptic localization of this enzyme was demonstrated by Couteaux and others (refs. in Couteaux, 1958, 1960). Fatt and Katz discovered that in a resting muscle, minute depolarizations occur spontaneously in the end-plate region of the fibre, and it was demonstrated by the effects of pharmacological agents that these result from the emission of small "parcels" or quanta of acetylcholine from the nerve ending (Fatt, Katz and del Castillo, refs. in Katz, 1962). It was suggested that each such "miniature end-plate potential" results from the discharge of acetylcholine from an axoplasmic synaptic vesicle into the synaptic gap across a reactive site on the presynaptic membrane, and that the effect of the nerve action potential is to increase greatly the rate at which this process occurs: "so that instead of an average of 1 ms we obtain two or three hundred units secreted within a fraction of 1 ms" (Katz, 1962) to produce the typical end-plate potential initiating the propagated depolarization (spike potential) of the muscle fibre membrane.

The comparative physiology of vertebrate and invertebrate muscular activity has been reviewed by Hoyle (1957). In insects and crustacea and in the slow (tonic) fibres of vertebrates, innervation is not confined to a single end-plate but is distributed in a multiterminal fashion over the fibre surface. In insect muscle, the arrival of a motor nerve initiates local areas of depolarization corresponding to the diffused neuromuscular synapses: an end-plate potential is set up at each junction and, in addition, an active membrane ("spike") response spreads decrementally from the region of the junction. In vertebrate fibres, application of curare blocks neuromuscular transmission by depressing the end-plate potential of the postsynaptic membrane below the threshold required for the initiation of a propagated response, and it is believed that curare specifically blocks the acetylcholine-mediated system. Similar application of curare has no effect on the insect neuromuscular junction, but agents with an analagous pharmacological action on the insect junction have been found: certain indole amines (5-hydroxy-tryptamine, etc.) depress the end-plate potential (and hence the active membrane response) and appear to act, as in the case of curare on the vertebrate ending, by blocking receptive sites on the postsynaptic surface normally available to the transmitter (Hill and Usherwood, 1961).

The acetylcholine-secreting nerve terminal in vertebrate motor end-plates is associated with a postsynaptic concentration of a specific

cholinesterase (Couteaux, 1960), and the non-cholinergic nature of the transmission mechanism in insects is corroborated by the absence of such esterase activity in the corresponding region of the insect neuromuscular junction (Wigglesworth, 1958).

Spontaneous miniature end-plate potentials, comparable in frequency and amplitude with those of vertebrate fibres, have been described by Usherwood (1961) in extensor tibiae muscles of *Schistocerca gregaria* and *Blaberus giganteus*. It is reasonable to suppose, therefore, that the mechanism by which quantal amounts of transmitter are deposited into the synaptic cleft is precisely similar in muscles of vertebrates and insects: in each case the immediate postsynaptic response takes the form of an end-plate potential. Variation in the overall electrical response to nervous stimulation shown by different types of fibre, which may be either "all-or-none" or graded, primarily relates to the pattern of subsequent membrane activity. The electron microscopic evidence is consistent with this, for the concentration of synaptic vesicles (visualized as containing the transmitter) within the terminal axoplasm, and the apposition of pre- and postsynaptic membranes, is essentially similar in vertebrate and insect endings. It thus appears that the activation of insect muscle is mediated by a physiological analogue of acetylcholine, sequestered in the nerve ending and thence supplied to the postsynaptic surface by a mechanism shared with the vertebrate neuromuscular junction.

The presence of large amounts of acetylcholine and cholinesterase (Corteggiani and Serfaty, 1939; Mikalonis and Brown, 1941; Tobias *et al.*,; Richards and Cutcomp, 1945) in the insect central nervous system suggests that although some other chemical transmitter is active at the neuromuscular junction, as in vertebrates at least part of the central synaptic transmission may be mediated by acetylcholine. However, as Roeder (1953) and Gilmour (1961) point out, the existence of this mechanism in insects has not yet been conclusively demonstrated. In mammalian ganglia, cholinesterase activity may occur in certain of the axons and cell bodies of the neurones and in the glial cytoplasm (Koelle, 1954; and refs. in Couteaux, 1958), while in the frog (Shen *et al.*, 1955) and in *Rhodnius* (Wigglesworth, 1958) most of this enzymatic activity is believed to be localized in the regions of synaptic contact. Wigglesworth reported that cholinesterase is confined to the glial cytoplasm, and is most evident in the neuropile; however, the details of its distribution amongst the finest glial and neuronal branches cannot be resolved with the light microscope. Similarly, in vertebrate ganglia the dimensions of the synaptic regions have so far precluded

histochemical recognition of the site of cholinesterase activity, so clearly seen at the vertebrate neuromuscular junction.

VIII. Conclusion

This account has shown that many of the specialized structural features of the insect nervous system appear to be reflected in its physiological organization. The lack of any circulatory system in the nervous system, for example, is correlated, in the cockroach at least, with a fibrous and cellular nerve sheath which, unlike its vertebrate counterpart (cf. Shanes, 1958), appears to be a relatively permeable structure. In this respect the insect nerve sheath is probably functionally more analogous to the vertebrate blood-brain barrier (Treherne, 1962d). The apparent permeability of the insect nerve sheath can also be correlated with the extensive extracellular system demonstrated in this insect, which ensures the accessibility of the necessary ions and molecules to the cells of the nervous system. The existence of a "cellular route" for the supply of nutrient materials to the neurones, *via* the perineurium and neurolglia, has also been postulated on the basis of the distribution of glycogen and lipids in the cockroach abdominal ganglion. It would be of obvious interest in future research to obtain a quantitative picture of the part these cells play in such trophic functions.

It has been pointed out that the relatively rapid movements of molecules across the neural lamella may be of significance in view of the extremely close contact of this structure with the cells of the fat body. The possibility exists that the metabolic processes of these cells may be intimately linked across the fibrous neural lamella and it is to be hoped that future investigations may throw some light on this hypothetical relationship.

The precise mechanism of synaptic transmission in insects still remains to be elucidated. It seems clear that the most promising line of further enquiry into the mode of synaptic transmission at the myoneural junction and in the central nervous system of insects lies in the identification of the transmitter substance or substances in central and peripheral synapses, and in the development of cytochemical methods for visualizing in the electron microscope the site of activity of the enzymes controlling the breakdown of these substances, as has already been accomplished in the case of the cholinesterase of the vertebrate motor end-plate.

Available structural information suggests that the organization of the neuromuscular junctions of insects and vertebrates are essentially

similar, and it appears, moreover, that the complexity of organization in the vertebrate central nervous system has been attained by topographical elaboration of basic structural units present in the insect system. In particular, while the chemistry of nervous transmission may vary between different groups of animals and between different regions of the nervous system of an individual, the comparative cytology of synaptic areas in insects and vertebrates appears to point to a common mechanism of synaptic excitation.

References

Andersson-Cedergren, E. (1959). *J. Ultrastructure Res.*, Suppl. **1,** 1–191. Ultrastructure of motor end plate and sarcoplasmic components of mouse skeletal muscle fiber as revealed by three-dimensional reconstructions from serial sections.

Ashhurst, D. E. (1959). *Quart. J. micr. Sci.* **100,** 401–12. The connective tissue sheath of the locust nervous system: a histochemical study.

Ashhurst, D. E. (1961a). *J. micro. Sci.* **102,** 399–405. The cytology and histochemistry of the neurones of *Periplaneta americana.*

Ashhurst, D. E. (1961b). *Quart. J. micr. Sci.* **102,** 455–61. A histochemical study of the connective tissue sheath of the nervous system of *Periplaneta americana.*

Ashhurst, D. E. (1961c). *Nature, Lond.* **191,** 1224–5. An acid mucopolysaccharide in cockroach ganglia.

Ashhurst, D. E. and Chapman, J. A. (1961). *Quart. J. micr. Sci.* **102**, 463–7. The connective tissue sheath of the nervous system of *Locusta migratoria:* an electron microscope study.

Baccetti, B. (1955). *Redia* **40,** 197–212. Richerche sulla fine struttura del perilemma nel sistema nervoso degli insetti.

Baccetti, B. (1956). *Redia* **41,** 259–76. Lo stroma di sostegno di organi degli insetti esaminato a luce polarizzata.

Baccetti, B. (1957). *Exp. Cell Res.* **13,** 158–60. Observations by polarized light on the supporting stroma of some organs in insects.

Baccetti, B. (1961a). *Atti. Accad. Torino* **95,** 343–50. Primi reperti sulla struttura submicroscopica dello stroma di sostegno di alconi organi degli insetti.

Baccetti, B. (1961b). *Redia* **46,** 1–7. Indagini comparative sulla ultrastruttura della fibrilla collagane nei diversi ordini degli insetti.

Beams, H. W. and King, R. L. (1932). *J. Morph.* **53,** 59–89. Cytoplasmic structures in the ganglion cells of certain Orthoptera, with special reference to the Golgi bodies, mitochondria, "vacuome", intracellular trabeculae (trophospongium) and neurofibrillae.

Beams, H. W., Sedar, A. W. and Evans, T. C. (1953). *Cellule* **55,** 293–304. Studies on the neurons of the grasshopper with special reference to the Golgi bodies, mitochondria and neurofibrillae.

Birks, R., Huxley, H. E. and Katz, B. (1960). *J. Physiol.* **150,** 134–68. The fine structure of the neuromuscular junction of the frog.

Bradfield, J. R. G. (1946). *Nature, Lond.* **157,** 876. Alkaline phosphatase in invertebrate sites of protein secretion.

Bradfield, J. R. G. (1950). *Proc. Linn. Soc. Lond.* **162,** 76–81. Biochemical aspects of cell morphology.

Brown, G. L., Dale, H. H. and Feldberg, W. (1936). *J. Physiol.* **87,** 394–424. Reaction of the normal mammalian muscle to acetylcholine and eserine.

Cajal, S. R. y. (1934). *Trab. Lab. Inv. biol. Univ. Madr.* **29,** 1–138. Les preuves objectives de l'unité anatomique des cellules nerveuses.

Cajal, S. R. y. and Sanchez, D. S. (1915). *Trab. Lab. Invest. biol. Univ. Madr.* **13,** 1–164. Contribución al conocimiento de los centros nerviosos de los insectos. Parte I. Retina y centros opticos.

Cerf, J. A., Grundfest, H., Hoyle, G. and McCann, F. V. (1959). *J. gen. Physiol.* **43,** 377–395. The mechanism of dual responsiveness in muscle fibers of the grasshopper *Romalea microptera.*

Chou, H. T. Y. and Meek, G. A. (1958). *Quart. J. micr. Sci.* **99,** 279–84. The ultrafine structure of lipid globules in the neurones of *Helix aspersa.*

Corteggiani, E. and Serfaty, A. (1939). *C.R. Soc. Biol. Paris,* **131,** 1124–27. Acetylcholine et cholinesterase chez les insectes et les arachnides.

Couteaux, R. (1958). *Exp. Cell. Res.,* suppl. **5,** 294–322. Morphological and cytochemical observations on the post-synaptic membrane at motor end-plates and ganglionic synapses.

Couteaux, R. (1960). "The Structure and Function of Muscle", I, Ch. 10. Academic Press, New York and London.

David, G. B., Mallion, K. B. and Brown, A. W. (1960). *Quart. J. micr. Sci.* **101,** 207–21. A method of silvering the "Golgi apparatus" (Nissl network) in paraffin sections of the central nervous system of vertebrates.

David, G. B. and Brown, A. W. (1961). *Quart. J. micr. Sci.* **102,** 391–97. The histochemical recognition of lipid in the cytoplasmic network of neurones of vertebrates.

Dale, H. H., Feldberg, W. and Vogt, M. (1936). *J. Physiol.* **86,** 353–80. Release of acetylcholine at voluntary motor nerve endings.

Dalton, A. J. (1960). "Symposium on Cell Physiology of Neoplasia". 14th Annual Symposium on Fundamental Cancer Research, p.161. Univ. Texas Press, Austin.

Dalton, A. J. (1961). *In* "The Cell", (ed. Brachet, J. and Mirsky, A. E.), Vol. 2, pp. 603–20. Academic Press, London and New York. Golgi apparatus and secretion granules.

Darwin, F. W. and Pringle, J. W. S. (1959). *Proc. roy. Soc.* B **151,** 194–203. The physiology of insect fibrillar muscle. I. Anatomy and innervation of the basalar muscle of lamellicorn beetles.

de Robertis, E. (1955). *Anat. Rec.* **121,** 284–5. Changes in the "synaptic vesicles" of the ventral acoustic ganglion after nerve section.

de Robertis, E. (1956). *J. Biophys. Biochem. Cytol.* **2,** 503–12. Submicroscopic changes of the synapse after nerve section in the acoustic ganglion of the guinea pig. An electron microscope study.

de Robertis, E. (1958). *Exp. Cell. Res.,* Suppl. 5, 347–69. Submicroscopic morphology and function of the synapse.

de Robertis, E. (1959). *Int. Rev. Cytol.* **8,** 61–96. Submicroscopic morphology of the synapse.

de Robertis, E. (1954). *Fed. Proc.* **13,** 35. Submicroscopic vesicular component in the synapse.

de Robertis, E. and Bennett, H. S. (1955). *J. Biophys. Biochem. Cytol.* **1,** 47–58. Some features of the submicroscopic morphology of synapses in frog and earth-worm.

Edwards, G. A. (1959). *J. Biophys. Biochem. Cytol.* **5,** 241–4. The fine structure of a multiterminal innervation of an insect muscle.

Edwards, G. A. (1960). *In* "Vierter Internationaler Kongress für Elektronenmikroskopie", **2,** Berlin, 1958. Vol. 2, 301–308. Springer, Berlin. Comparative studies on the fine structure of motor units.

Edwards, G. A., Ruska, H. and de Harven, E. (1958a). *J. Biophys. Biochem. Cytol.* **4,** 107–14. Electron microscopy of peripheral nerves and neuromuscular junctions in the wasp leg.

Edwards, G. A., Ruska, H. and de Harven, E. (1958b). *J. Biophys. Biochem. Cytol.* **4,** 251–6. Neuromuscular junctions in the flight and tymbal muscles of the Cicada.

Eichner, D. (1956). *Z. Zellforsch.* **43,** 501–12. Zur fräge der neurofibrillen.

Feng. T. P. (1941). *Biol. Symp.* **3,** 121–52. The local activity around the skeletal N-M junctions produced by nerve impulses.

Frankenhaeuser, B. and Hodgkin, A. L. (1956). *J. Physiol.* **131,** 341–76. The after-effects of impulses in the giant nerve fibres of *Loligo.*

Furchpan, E. J. and Potter, D. D. (1959). *J. Physiol.* **148,** 289–325. Transmission of the giant motor synapses of the cockroach.

Geren, B. B. and Schmitt, F. O. (1954). *Proc. nat. Acad. Sci., Wash.* **40,** 863–70. The structure of the Schwann cell and its relation to the axon in certain invertebrate nerve fibers.

Gilmour, D. (1961). "The Biochemistry of Insects", Ch. 5. Academic Press, New York and London.

Gray, E. G. (1959). *Proc. roy. Soc.* B, **150,** 233–9. Electron microscopy of collagen-like connective tissue fibrils of an insect.

Gray, E. G. (1960). *Phil. Trans.* B, **243,** 77–94. The fine structure of the insect ear.

Gresson, R. A. R., Threadgold, L. T. and Stinson, N. E. (1956). *Cellule* **58,** 7–16. The Golgi elements of the neurones of *Helix*, *Locusta* and *Lumbricus.*

Golgi, C. (1898). *Arch. ital. Biol.* **30,** 60–71. Sur le structure des cellules nerveuses.

Hess, A. (1958a). *J. Biophys. Biochem. Cytol.* **4,** 731–42. The fine structure of nerve cells and fibers, neuroglia, and sheaths of the ganglion chain in the cockroach (*Periplaneta americana*).

Hess, A. (1958b). *Quart. J. micr. Sci.* **99,** 333–40. The fine structure and morphological organization of the peripheral nerve-fibres and trunks of the cockroach (*Periplaneta americana*).

Hill, R. B. and Usherwood, P. N. R. (1961). *J. Physiol.* **157,** 393–401. The action of 5-hydroxytryptamine and related compounds on neuromuscular transmission in the locust *Schistocerca gregaria.*

Hirsch, G. C. (1939). *Protoplasma-Monogr., Berl.* **18.** Form und Stoffwechsel der Golgi-Körper.

Hirsch, G. C. (1962). *In* "Handbuch der Biologie", **1.** Akademische Verlagsgesellschaft Athenaion, Konstanz. Das Lamellen—Vakuolen—Feld.

Hodgkin, A. L. and Keynes, R. D. (1955). *J. Physiol.* **128,** 61–88. The potassium permeability of a giant nerve fibre.

Hoyle, G. (1952). *Nature, Lond.* **169,** 281–2. High blood potassium in insects in relation to nerve conduction.

Hoyle, G. (1953). *J. exp. Biol.* **30,** 121–35. Potassium ions and insect nerve muscle.

Hoyle, G. (1957). "The Nervous Control of Muscular Contraction". Cambridge University Press.

Johansson, A. S. (1957). *Trans. Amer. ent. Soc.* **83,** 119–83. The nervous system of the Milkweed Bug, *Oncopeltus fasciatus* (Dallas).

Kantor, T. G. and Schubert, M. (1957). *J. histochem. Cytol.* **5,** 28–32. The difference in permeability of cartilage to cationic and anionic dyes.

Katz, B. (1962). *Proc. roy. Soc.* B, **155,** 455–77. The Croonian lecture. The transmission of impulses from nerve to muscle, and the subcellular unit of synaptic action.

Keonig, H. (1958). *J. Biophys. Biochem. Cytol.* **4,** 785–92. An autogradiographic study of nucleic acid and protein turnover in the mammalian neuraxis.

Keynes, R. D. and Lewis, P. R. (1951). *J. Physiol.* **114,** 151–82. The sodium and potassium content of cephalopod nerve fibres.

Koelle, G. B. (1954). *J. comp. Neurol.* **100,** 211–35. The histochemical localization of cholinesterase in the central nervous system of the rat.

Koelle, G, B. and Friedenwald, J. S. (1949). *Proc. Soc. exp. Biol. Med.* **70,** 617–22. A histochemical method for localizing cholinesterase activity.

Lundberg, A. (1951). *Acta physiol. scand.* **22,** 365–75. On the ability of some cations to inhibit the potassium-depolarization of frog nerve fibres.

Maddrell, S. H. P. (1962a). *Nature, Lond.* **194,** 605–6. A diuretic hormone in *Rhodnius prolixus* Stal.

Maddrell, S. H. P. (1962b). *Ph.D. Thesis, University of Cambridge.*

Malhotra, S. K. (1956). *Quart. J. micr. Sci.* **97,** 177–86. The cytoplasmic inclusions of the neurones of certain insects.

Malhotra, S. K. (1959). *Quart. J. micr. Sci.* **100,** 339–67. What is the "Golgi apparatus" in its classical site within the neurones of vertebrates?

Malhotra, S. K. (1960). *Quart. J. micr. Sci.* **101,** 69–74. The Nissl–Golgi complex in the Purkinje cells of the tawny owl, *Strix aluco.*

Malhotra, S. K. (1961). *Quart. J. micr. Sci.* **102,** 387–9. Colouration of the Golgi–Nissl network in a vertebrate neurone by sudan black.

Malhotra, S. K. and Meek, G. A. (1960). *Quart. J. micr. Sci.* **101,** 389–94. The electron microscopy of the "Golgi apparatus" in the Purkinje cells of owls.

Manegold, E. (1929). *Kolloidzschr.* **49,** 372–95. Die Dialyse durch Kollodiummembranen und der Zusammenhang Zwischen Dialyse, Duffusion und Membranstruktur.

Mikalonis, S. J. and Brown, R. H. (1941). *J. cell. comp. Physiol.* **18,** 401–2. Acetylcholine and cholinesterase in the insect central nervous system.

Monti, R. (1913). *Arch. Anat. Zool.* **15,** 349–433. Sur les relations mutuelles entre les elements dans le systeme nerveux central des insectes.

Moussa, T. A. and Banwahy, M. (1959). *Nature, Lond,* **184,** 915–6. Effect of insecticides on the bodies of Nissl and neurofibrillae of the locust, *Schistocerca gregaria.*

Narahashi, T. and Yamasaki, T. (1960). *J. Physiol.* **151,** 75–88. Mechanism of the after-potential production in the giant fibres of the cockroach.

Nath, V. (1926). *Biol. Rev.* **2,** 52–79. On the present position of the mitochondria and the Golgi apparatus.

Palade, G. E. (1954). *Anat. Rec.* **118,** 335–6. Electron microscope observations of inter neuronal and neuromuscular synapses.

Palay, S. L. (1954). *Anat. Rec.* **118,** 336–7. Electron microscope study of the cytoplasm of neurons.

Palay, S. L. (1958). *Exp. Cell Res.* suppl. 5, 275–93. The morphology of synapses in the central nervous system.

Palay, S. L. and Palade, G. E. (1955). *J. Biophys. Biochem. Cytol.* **1,** 69–88. The fine structure of neurons.

Palay, S. L., McGee-Russell, S. M., Gordon, S. and Grillo, M. (1962). *J. Biophys. Biochem. Cytol.* **12,** 385–410. Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide.

Parat, M. and Painlevé, J. (1924a). *C. R. Acad. Sci., Paris* **179,** 543–4. Constitution du cytoplasme d'une cellule glandulaire: le cellule des glandes salivaires de la larve du Chironome.

Parat, M. and Painlevé, J. (1924b). *C. R. Acad. Sci., Paris* **179,** 612–4. Observation vitale d'une cellule glandulaire en activité. Nature et role de l'appareil rèticulaire interne de Golgi et de l'appareil de Holmgren.

Parat, M. and Painlevé, J. (1924c). *C. R. Acad. Sci., Paris* **179,** 844–6. Appareil reticulaire interne de Golgi, trophosponge de Holmgren et vacuome.

Pipa, R. L. (1961). *J. comp. Neurol.* **116,** 15–22. Studies on the hexapod nervous system. III. Histology and histochemistry of cockroach neuroglia.

Pipa, R. L. and Cook, E. F. (1958). *J. Morph.* **103,** 353–85. The structure and histochemistry of the connective tissue of the sucking lice.

Pipa, R. L., Cook, E. F. and Richards, A. G. (1959). *J. comp. Neurol.* **113,** 401–23. Studies on the hexapod nervous system. II. The histology of the thoracic ganglion of the adult cockroach, *Periplaneta americana.*

Pipa, R. L., Nishioha, R. S. and Bern, H. A. (1962). *J. Ultrastructure Res.* **6,** 164–70. Studies on the hexapod nervous system. V. The Ultrastructure of cockroach gliosomes.

Pringle, J. W. S. (1939). *J. exp. Biol.* **16,** 220–31. The motor mechanism of the insect leg.

Pringle, J. W. S. (1957). *In* "Recent Advances in Invertebrate Physiology", (ed. Scheer, B.T.). University of Oregon Publications, Eugene, Oregon.

Rebhun, L. I. (1956). *J. Biophys. Biochem. Cytol.* **2,** 93–104. Electron microscopy of basophilic structures of some invertebrate oocytes. I. Periodic lamellae and the nuclear envelope.

Reger, J. F. (1958). *Anat. Rec.* **130,** 7–14. The fine structure of neuromuscular synapses of gastrocnemii from mouse and frog.

Reger, J. F. (1961). *J. Biophys. Biochem. Cytol.* 10 suppl., 111–21. The fine structure of neuromuscular junctions and the sarcoplasmic reticulum of extrinsic eye muscle of *Fundulus heteroclitus.*

Reger, J. F. (1959). *J. Ultrastructure Res.* **2,** 269–82. Studies on the fine structure of normal and denervated neuromuscular junctions from mouse gastrocnemius.

Revel, J. P. (1962). *J. Biophys. Biochem. Cytol.,* **12,** 571–88. The sarcoplasmic reticulum of the bat cricothyroid muscle.

Revel, J. P., Napolitano, L. and Fawcett, D. W. (1960). *J. Biophys. Biochem. Cytol.* **8,** 575–589. Indentification of glycogen in electron micrographs of thin tissue sections.

Richards, A. G. and Cutcomp. L. K. (1945). *J. cell. comp. Physiol.* **26,** 57–61. The cholinesterase of insect nerves.

Richards, A. G. and Schneider, D. (1958). *Z. Naturf.* **13,** 680–7. Über den komplexen Bau der Membranen des Bindegewebes von Insekten.

Robertson, J. D. (1956). *J. Biophys. Biochem. Cytol.* **2,** 369–80. Some features of the ultrastructure of reptilian skeletal muscle.

Roeder, K. D. (1953). "Insect Physiology", Chap. 17. John Wiley & Sons, New York.

Rosenbluth, J. (1962). *J. Biophys. Biochem. Cytol.* **12,** 329–59. The fine structure of acoustic ganglion in the rat.

Rudall, K. M. (1955). *Symp. Soc. exp. Biol.* **9,** 49–71. The distribution of collagen and chitin.

Scharrer, B. C. J. (1939). *J. comp. Neur.* **70,** 77–88. The differentiation between neuroglia and connective tissue sheath in the cockroach (*Periplaneta americana*).

Scharrer, B. C. J. (1961). *Z. Zellforsch.* **54,** 530–40. Neurosecretion XII. The formation of neurosecretory granules in the earthworm, *Lumbricus terrestris* L.

Schmitt, F. O., Gross, J. and Highberger, J. H. (1955). *Symp. Soc. exp. Biol.* **9,** 148–62. States of aggregation of collagen.

Shafiq, S. A. (1953). *Quart. J. micr. Sci.* **95,** 305–14. Cytological studies on the neurones of *Locusta migratoria.* Part 11. Cytoplasmic inclusions during the differentiation and growth of nerve cells.

Shafiq, S. A. and Casselman, W. G. B. (1954). *Quart. J. micr. Sci.* **95,** 315–20. Cytological studies of the neurones of *Locusta migratoria.* Part III. Histochemical investigations, with special reference to lipochondria.

Shanes, A. M. (1951). *J. gen. Physiol.* **34,** 795–807. Potassium movement in relation to nerve activity.

Shanes, A. M. (1958). *Pharm. Rev.* **10,** 61–164. Electrochemical aspects of physiological and pharmacological action in excitable cells. I. The resting cell and its alteration by extrinsic factors.

Shen, S. C., Greenfield, P. and Boell, E. J. (1955). *J. comp. Neurol.* **102,** 717–743. The distribution of cholinesterase in the frog brain.

Smith, D. S. (1960). *J. Biophys. Biochem. Cytol.* **8,** 447–66. The innervation of the fibrillar flight muscle of an insect: *Tenebrio molitor.*

Smith, D. S. (1961a). *J. Biophys. Biochem. Cytol.* **10** suppl., 123–58. The structure of insect fibrillar flight muscle.

Smith, D. S. (1961b). *J. Biophys. Biochem. Cytol.* **11,** 119–44. The organization of the flight muscle in a dragonfly, *Aeshna* sp.

Smith, D. S. and Wigglesworth, V. B. (1959). *Nature, Lond.* **183,** 127–8. The occurrence of collagen in the perilemma of insect nerve.

Stämpfli, R. and Nishie, K. (1956). *Helv. physiol. acta* **14,** 93–104. Effects of calcium-free solutions on membrane potential of myelinated nerve fibres of the Brazilian frog *Leptodactylus ocellatus.*

Tobias, J. M., Kollros, J. J. and Savit, J. (1946). *J. cell. comp. Physiol.* **28,** 159–82. Acetylcholine and related substances in the cockroach, fly and crayfish and the effect of DDT.

Treherne, J. E. (1960). *J. exp. Biol.* **37,** 513–33. The nutrition of the central nervous system in the cockroach, *Periplaneta americana.* The exchange and metabolism of sugars.

Treherne, J. E. (1961a). *J. exp. Biol.* **38,** 315–22. Sodium and potassium fluxes in the abdominal nerve cord of the cockroach, *Periplaneta americana.*

Treherne, J. E. (1961b). *J. exp. Biol.* **38,** 629–36. The movements of sodium ions in the isolated abdominal nerve cord of the cockroach, *Periplaneta americana.*

Treherne, J. E. (1961c). *Nature, Lond.* **191,** 1223–4. Exchange of sodium ions in the central nervous system of an isect.n

Treherne, J. E. (1961d). *J. exp. Biol.* **38,** 729–36. The efflux of sodium ions from the last abdominal ganglion of the cockroach, *Periplaneta americana.*

Treherne, J. E. (1961e). *J. exp. Biol.* **38,** 737–46. The kinetics of sodium transfer in the central nervous system of the cockroach, *Periplaneta americana.*

Treherne, J. E. (1962a). *Nature, Lond.* **193,** 750–2. Distribution of water and inorganic ions in the central nervous system of an insect (*Periplaneta americana*).

Treherne, J. E. (1962b). *J. exp. Biol.* **39,** 193–217. The distribution and exchange of some ions and molecules in the central nervous system of *Periplaneta americana.*

Treherne, J. E. (1962c). *J. exp. Biol.* **39,** 631–41. Some effects of the ionic composition of the extracellular fluid on the electrical activity of the cockroach abdominal nerve cord.

Treherne, J. E. (1962e). *Nature, Lond.* **196,** 1181–3. Transfer of substances between the blood and central nervous system in vertebrate and invertebrate animals.

Tristam, G. R. (1953). *In* "The Proteins", 1, p.224 (ed. H. Neurath and K. Bailey). Academic Press, New York and London. The amino-acid composition of proteins.

Trujillo-Cenóz, O. (1959). *Z. Zellforsch.* **49,** 432–46. Study on the fine structure of the central nervous system of *Pholus labruscoe* (Lepidoptera).

Trujillo-Cenóz, O. (1962). *Z. Zellforsch.* **56,** 649–82. Some aspects of the structural organization of the anthropod ganglia.

Twarog, B. M. and Roeder, K. D. (1956). *Biol. Bull., Woods Hole* **111,** 278–86. Properties of the connective tissue sheath of the cockroach abdominal nerve cord.

Usherwood, P. N. R. (1961). *Nature, Lond.* **191,** 814–5. Spontaneous miniature potentials from insect muscle fibres.

Webster, H. de F. (1962). *J. Biophys. Biochem. Cytol.* **12,** 361–383. Transient, focal accumulation of axonal mitochondria during the early stages of Wallerian degeneration.

Wigglesworth, V. B. (1942). *J. exp. Biol.* **19,** 56–77. The storage of protein, fat, glycogen and uric acid in the fat body and other tissues of the mosquito larvae.

Wigglesworth, V. B. (1953). "The Principles of Insect Physiology", 4th edn., Ch. 5. Methuen, London.

Wigglesworth, V. B. (1956). *Quart. J. micr. Sci.* **97,** 89–98. The haemocytes and connective tissue formation in an insect *Rhodnius prolixus* (Hemiptera).

Wigglesworth, V. B. (1957). *Proc. roy. Soc.* B, **147,** 185–99. The use of osmium in the fixation and staining of tissues.

Wigglesworth, V. B. (1958). *Quart. J. micr. Sci.* **99,** 441–50. The distribution of esterase in the nervous system and other tissues of the insect *Rhodnius prolixus.*

Wigglesworth, V. B. (1959a). *Quart. J. micr. Sci.* **100,** 285–98. The histology of the nervous system of an insect, *Rhodnius prolixus* (Hemiptera). I. The peripheral nervous system.

Wigglesworth, V. B. (1959b). *Quart. J. micro. Sci.* **100,** 299–313. The histology of the nervous system of an insect, *Rhodnius prolixus* (Hemiptera). II. The central ganglia.

Wigglesworth, V. B. (1960a). *J. exp. Biol.* **37,** 500–12. The nutrition of the central nervous system of the cockroach, *Periplaneta americana.* The mobilization of reserves.

Wigglesworth, V. B. (1960b). *Quart. J. micr. Sci.* **101,** 381–8. Axon structure and the dictyosomes (Golgi bodies) in the neurones of the cockroach *Periplaneta americana.*

Yamasaki, T. and Narahashi, T. (1959). *J. insect Physiol.* **3,** 146–58. The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon.

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